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Full Length Research Paper

Integrated management of *Aspergillus* species and aflatoxin production in groundnut (*Arachis hypogaea* L.) through application of farm yard manure and seed treatments with fungicides and *Trichoderma* species

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Groundnut (*Arachis hypogaea* L.) is an important cash crop in the lowland areas of Ethiopia. However, prevalence of *Aspergillus* invasions and subsequent aflatoxin contamination compromises the quality of groundnut kernels. This study was conducted to evaluate the effect of farm yard manure (FYM) and seed treatments against *Aspergillus* species pod colonization and aflatoxin accumulation under field conditions. The inhibitory efficacy of *Trichoderma* species as biocontrol agents was also assessed. A total of 20 treatment combinations including pre-planting applications of FYM at 0, 2.5, 5, and 7.5 tons/ha and seed treatments with carbendazim at 2 g/kg and mancozeb at 3 g kg^{-1}, and *Trichoderma harzianum* and *Trichoderma viride* each at 5 g/kg as well as untreated seed as control were used. Treatments were laid out in a randomized complete block design (RCBD) in three replications. The experiment was conducted in two consecutive seasons (2014 and 2015) at Babile Haramaya University sub-Research Station. The highest pod and seed yields (1901.5 and 1281.5 kg/ha, respectively) were recovered from plots treated with *T. harzianum* at 5 g/kg seed. *A. flavus* was abundantly recorded in control plots, which could be responsible for the high aflatoxin B_1 (5704.4 µg/kg) and B_2 (2219.0 µg/kg) contamination. However, plots treated with *T. harzianum* at 5 g/kg seed and FYM at 5 tons/ha + *T. harzianum* at 5 g/kg were free from aflatoxin. Integrations of *T. harzianum* as biocontrol seed treatment and soil amendment with FYM were effective in the pre-harvest management of *Aspergillus* spp. and aflatoxins contamination.

Key words: Aflatoxin, *Aspergillus,* carbendazim, farm yard manure, groundnut, mancozeb, *Trichoderma* species.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important monoecious annual legume used as oilseed, food and animal feed all over the world (Upadhyaya et al., 2006). In Ethiopia, groundnut production is adapted to warm climates and predominantly grown under rainfed conditions (Amare and Tamado, 2014). The total groundnut yield in the country was 1,296,364.18 tons for 2016/2017, with the productivity of 1.73 tons/ha (CSA, 2016/2017). Eastern and lowland part of the country, mainly East Hararghe zone, is the leading groundnut production area accounting for 43.4% of the total production. In this area, groundnut is replacing major crops like maize and sorghum (Amare and Tamado, 2014). Despite its ever-increasing importance, groundnut quality and marketability are hampered by pre- and post-harvest aflatoxin contamination in the area (Ayalew et al., 1995; Eshetu, 2010; Chala et al., 2013; Mohammed and Chala, 2014; Mohammed et al., 2016).

Groundnut is one of the legume crops most susceptible to invasion by *Aspergillus flavus* and *Aspergillus parasiticus*, which subsequently produce aflatoxin, a mycotoxin that poses serious human and animal health risks (Williams et al., 2004). Aflatoxin is a major constraint to groundnut export and foreign exchange for many countries in sub-Saharan Africa such as Ethiopia and Kenya. Losses from rejected shipments and lower prices for poor quality grain can devastate a developing country's export markets (IFPRI, 2003). These losses can have a higher impact on sub-Saharan Africa due to the favorable environmental conditions for the growth of mycotoxigenic fungi and the lack of adequate storage infrastructure. For example, in Nigeria, regulatory agencies destroyed mycotoxin-contaminated foods worth more than US$ 200, 000 in 2010 (Hussaini, 2013). Income losses due to aflatoxin contamination cost US producers more than US$ 100 million per year, on average, including US$ 26 million paid to peanut farmers alone (US$ 69.34 ha^{-1}) (Coulibaly et al., 2008).

In Ethiopia, aflatoxin contamination of groundnut is often reported. Ayalew et al. (1995) reported a total aflatoxin contamination in groundnut ranged from 5 to 250 µg/kg, furthermore Eshetu (2010) reported aflatoxin level up to 447 µg/kg in groundnut seed from Eastern Ethiopia. Death cases in Kenya were reportedly caused by ingestion of maize with aflatoxin concentrations up to 4,400 µg/kg (Azziz-Baumgartner et al., 2005). However, Chala et al. (2013) reported aflatoxin levels of about 12,000 µg/kg in groundnut seed from Babile district in Eastern Ethiopia orders of magnitude higher than the levels observed in Kenya; whereas the acceptable limit set for the European Union is 4 µg/kg (OJEU, 2010). However, there is no acceptable limit of aflatoxin in Ethiopia. Recently, Mohammed et al. (2016) reported aflatoxin B_1 concentrations of 2,526 and 158 µg/kg, in groundnut seed and groundnut cake locally known as "*Halawa*", respectively, from Eastern Ethiopia. The high aflatoxin levels observed indicate the urgent need for management of *Aspergillus* and associated aflatoxin contamination in this area.

Management practices that reduces the incidence of aflatoxin contamination at pre-harvest in the field include timely planting, maintaining optimal plant densities, proper plant nutrition, avoiding drought stress, controlling other plant pathogens, weeds and insect pests and proper harvesting (Bruns, 2003). The application of lime, FYM, poultry manure, host plant resistance, and chemical fumigation of the soil was employed earlier in reducing the aflatoxin contamination in the groundnut crops (ICRISAT, 2000). Among which, lime, FYM and cereal crop residues as soil amendments have shown to be effective in reducing *A. flavus* contamination as well as aflatoxin levels by 50 to 90% (Bruns, 2003). Besides biocontrol agents such as, non-toxigenic bacterial strains, especially *Bacillus* species (Bottone and Peluso, 2003) and the fungus *Trichoderma harzianum* (Inglis and Kawchuk, 2002) have also been used. Most of these biocontrol options are not accessible or are difficult to apply on the seeds for smallholder farmers in developing countries. Hence, there is a need for evaluations of effective integrations of biocontrol agents with cultural practices that are affordable for adoption by smallholder farmers.

Fungicide seed treatment is also beneficial for the management of seed-borne pathogens and its application before planting decreases pre-emergence as well as post-emergence damping-off and increases seedling survival rates or establishment and plant vigor in various crops (Elwakil and El-Metwally, 2000). In Eastern Ethiopia, Getnet et al. (2013) conducted field experiments aimed at suppressing aflatoxigenic fungi on groundnut through fungicide seed treatments with mancozeb and carbendazim and reported seed yield increase by 42.1 and 70.9%, respectively. However, the comparison of fungicide and biocontrol seed treatments with cultural practices in reducing the pre-harvest *Aspergillus* spp. invasion and aflatoxin contamination of groundnut was meager in the area.

Given the negative effects that aflatoxins have on human health and marketability of groundnut, it is imperative to find cultural practices that can help reduce/eliminate aflatoxins from this crop, integration with biological control methods adds the advantage of being eco-friendly and relatively safe. The objectives of this study were to evaluate the effects of FYM and seed

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Table 1. Description of treatments tested to manage *Aspergillus* species aflatoxin production in groundnut ~~in~~ under field experiments.

No.	Treatment	No.	Treatment
T1	Negative control	T11	FYM 2.5 tons/ha + *T. harzianum* 5 g/kg
T2	FYM 2.5 tons/ha	T12	FYM 2.5 tons/ha + *T. viride* 5 g/kg
T3	FYM 5 tons/ha	T13	FYM 5 tons/ha + Carbendazim 2 g/kg
T4	FYM 7.5 tons/ha	T14	FYM 5 tons/ha + Mancozeb 3 g/kg
T5	Carbendazim 2 g/kg	T15	FYM 5 tons/ha + *T. harzianum* 5 g/kg
T6	Mancozeb 3 g/kg	T16	FYM 5 tons/ha + *T. viride* 5 g/kg
T7	*T. harzianum* 5 g/kg	T17	FYM 7.5 tons/ha + Carbendazim 2 g/kg
T8	*T. viride* 5 g/kg	T18	FYM 7.5 tons/ha + Mancozeb 3 g/kg
T9	FYM 2.5 tons/ha + Carbendazim 2 g/kg	T19	FYM 7.5 tons/ha + *T. harzianum* 5 g/kg
T10	FYM 2.5 tons/ha + Mancozeb 3 g/kg	T20	FYM 7.5 tons/ha + *T. viride* 5 g/kg

[a]FYM: Farm yard manure.

treatments with fungicides and biocontrol agents on the development of *Aspergillus* spp. and aflatoxin production under field conditions.

MATERIALS AND METHODS

Field experiment and treatment applications

Experiments were conducted at Babile research sub-station of Haramaya University during 2014 and 2015 major cropping seasons and planted in mid-May each year under rainfed conditions. The site is located in eastern Hararghe zone at 9°13'13.5'' N and 42°19'20.9'' E with an altitude of 1647 m above sea level. The growing season starts from mid-April to end of October and the area has an annual average rainfall of 569 mm. The soil at Babile research sub-station is sandy loam soils.

The local groundnut variety *Oldhale*, commonly grown in Babile, Gursum and Bisidmo areas by smallholder farmers (Bethlehem, 2011) was used as experimental material. Treatments included soil amendments with farm yard manure (FYM) at rates of 0, 2.5, 5, and 7.5 tons/ha applied one week before planting. Fungicides seed treatments with carbendazim at 2 g/kg and mancozeb at 3 g/kg seed; biocontrol agents *T. harzianum* and *Trichoderma viride* each at 5 g/kg seed; and untreated seeds used as a control.

Farm yard manure (FYM) was a mixture of cattle and goat manure collected for approximately 10 years and protected from water erosion commonly used by growers as organic fertilizer. It is rich in nutrients, including trace elements necessary for crop growth and inducing resistance in the plants. The amount of FYM required for the experiment was purchased from the growers and spreaded on the soil surface and amended to the soil manually a week prior to planting the seeds. Agronomic practices were performed for manual hand weeding and inter-cultivations in each plot. Groundnut harvesting at physiological maturity was performed manually using human labors in avoiding the mechanical damages of the pods. Fungicide seed treatments were done by first weighing each chemical and placing it in a 100 mL Erlenmeyer flask to which the groundnut seeds were added; this was followed by 1-h shaking (Flask shaker SF1, Stuart Scientific, UK) at speed of 150 rpm to uniformly coat the seeds. A total of 20 treatment combinations (Table 1) replicated three times were set up in a factorial RCBD. The crop was planted 60 cm between rows and 10 cm apart within rows giving a total of 150 plants per plot. Each plot had 5 rows of 30 plants per row 3 × 3 m plots. Plants in the central three rows were used for data collection.

For the *Trichoderma* species treatment application, pure isolates of *T. harzianum* and *T. viride* preserved at Ambo Plant Protection Research Center (Ambo Ethiopia), were used. Isolates were multiplied using PDA medium. Seeds were treated with *Trichoderma* from 8-day-old PDA culture. Mycelial mat/harvesting (at 5 g/kg seed for each species) with spores and conidia amended with carboxy-methyl cellulose (CMC) (0.5%) placed in 1000 ml Erlenmeyer flask with droplets of sterile distilled water to produce a thin paste. Then the seeds were mixed through rotary shaker at 150 rpm for 6 h. Control seeds were treated with sterile distilled water. Seeds were incubated at 25°C for 24 h to create fungal emergence for further effective adherence.

The variables observed were: days to 50% emergence (D50%E), days to 50% flowering (D50%F), and days to 95% maturity (D95%M) of groundnut. Plant populations were evaluated as stand count at emergence (SCE) and stand count at harvest (SCH). Yield components measured were: number of pods per plant (NPP), seeds per pod (NSP), pod yield (PY), seed yield (SY), the 100 seed weight (HSW), and shelling percentage (SHP) of pods. The crop was harvested at physiological maturity and dried on sterilized materials (rugs). Each treatment was harvested and groundnut seed sample of 1 kg homogeneously produced from each plot and a total of 120 samples in two cropping seasons were taken for further laboratory analysis.

Isolation and identification of *Aspergillus* spp. from groundnut seeds

Aspergillus spp. were isolated from harvested groundnut seed samples using modified Dichloran Rose Bengal (MDRB) (Horn and Dorner, 1998). Twenty grams of groundnut seeds were weighed from each sample and placed in 50 mL Falcon™ tubes containing 25 ml of sterile distilled water. From each sample, 50 and 100 µL suspensions were spread on MDRB medium and incubated at 37°C for 72 h. Colonies of *Aspergillus* spp. were counted on MDRB medium. Fungal species load per treatment was derived from plate counts, expressed as the logarithm of the number of colony forming unit (CFU) and were presented as Log_{10} CFU/g.

Spores from the individual colony were aseptically transferred to fresh MDRB plates using a sterile needle. Small pieces of agar containing hyphal tip were transferred to Czapek Dox Agar (CDA;

OXOID Ltd, Hampshire, England), slant medium prepared according to Horn et al. (1996) and incubated at 30°C for 10 to 14 days for identification. Isolates were identified using taxonomic systems of *Aspergillus* (Klich, 2002) and confirmation was done by comparison with reference cultures of Dr. Bruce Horn's collection (USDA National Peanut Research Laboratory, Dawson, Georgia, USA).

Aflatoxin analysis from groundnut seed

About 1 kg of groundnut sample from each plot was taken and totally produces 120 samples, and representative of 50 g of seed was suspended in 1 L glass jar (Waring Products Div., Torrington, CT, USA) with 100 mL of methanol/distilled water (80/20 v/v, respectively) and blended at high speed (13, 000 rpm) for 1 min, for aflatoxin extraction. A pre-pleated filter paper Whatman No. 4 was inserted in the mixture, 500 µL of filtrate was transferred to a disposable glass test tube followed by addition of 500 µL of acetonitrile to the same tubes and mixed thoroughly (Mohammed et al., 2016). Then, 500 µL of the mixture was pipetted into the 1.5-mL columns prepared for cleaning. The eluate containing aflatoxins was collected into 500 µL-ultra performance liquid chromatography (UPLC) glass vials and immediately closed with caps with septa. The limit of detection (LOD) for aflatoxins was 1 µg/kg for B_1 and G_1 and 0.05 µg/kg for aflatoxin B_2 and G_2. Aflatoxin standards of B_1, B_2, G_1, and G_2 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Stock and spike solutions of each aflatoxin B_1, B_2, G_1, and G_2 were prepared according to the protocol developed by Sobolev and Dorner (2002).

Aflatoxin detection was carried out by UPLC Acquity, using column ACQUITY UPLC ® BEH C18 1.7 µm, 2.1 × 50 mm (Waters, Milford, MA, USA), at a temperature of 40°C, and with fluorescence detection. The mobile phase was methanol/water/acetonitrile (20/70/10%, v/v/v, respectively), and the flow rate of 0.25 mL/min with an injection volume of 1 µL was used. All instrument control, analysis, and data processing were performed using Waters® Empower 3® Chromatography Data Software (CDS). The concentration of each class of aflatoxin was computed as µg/kg.

Data analysis

Data collected from the field and laboratory experiments were subjected to analysis of variance (ANOVA) using general linear models (GLM) of SAS (2002) for Windows 9 (SAS Institute Inc., Cary, NC, USA). Means were compared using the least significant difference (LSD) at the $p \leq 0.05$ level of significance. Correlation analysis was done using Pearson correlation coefficient method (Pelosi and Sandifer, 2003).

RESULTS

Efficacies of treatments on *Aspergillus* spp. contamination in groundnut

The experiments were employed for the comparison of fungicides and *Trichoderma* spp. seed treatment as biological control agent integration with FYM soil application against pre-harvest *Aspergillus* spp. invasion and subsequent aflatoxin production. In the 2014 experiment, the highest seed invasion of *A. flavus* (1.3 log CFU/g) and *A. parasiticus* (0.48 log CFU/g) was found in samples from plots treated with FYM at 5 tons/ha followed by control plot samples infected with *A. flavus* of 1.08 log CFU/g (Table 2). Among plots that received sole FYM treatment, the lowest seed invasion (0.69 log CFU/g) of *A. flavus* was recorded at the highest rate of FYM at 7.5 tons/ha soil application. However, plots sown with seeds treated with carbendazim at 2 g/kg and *T. harzianum* 5 g/kg were devoid of *Aspergillus* spp. invasion, which is 100% reduction in seed invasion compared with the control plots in 2014 season. On the other hand, *A. niger* occurrences were relatively less with the highest (0.78 log CFU/g) of seed invasion in plots treated with FYM 2.5 tons/ha + *T. harzianum* 5 g/kg. The samples obtained from plots treated with FYM 2.5 tons/ha integrated with mancozeb 3 g/kg and *T. viride* 5 g/kg and FYM 5 tons/ha + *T. harzianum* 5 g/kg also had less contamination by *Aspergillus* spp.

In the samples from the 2015 experiment, the highest seed invasion of *A. flavus* (1.63 log CFU/g) was found in control plots, followed by plots sown with seeds treated with mancozeb 3 g/kg which had 1.36 log CFU/g (Table 2). Among the plots sown with seed treatments, *T. harzianum* 5 g/kg managed 100% seed invasion by *Aspergillus* spp., except *Aspergillus niger*, which was recorded at 0.3 log CFU/g of seed. Plots treated with *T. harzianum* 5 g/kg in single and combination with FYM at 5 tons/ha had no or relatively less invasion with *Aspergillus* spp., which is consistent with the preceding year results.

Effects of treatments on aflatoxin contamination of groundnut seed

In 2014 cropping season, samples (n=60), 10% (n= 6) were positive for aflatoxin contaminations, while 90% (n= 54) were negative (Table 3). Among positive samples, 33% had aflatoxin B_1 below 10 µg/kg, which is the tolerable limit of East African Commission. However, the highest aflatoxin concentration of B_1 (1340.6 µg/kg) was detected in samples from plots treated with FYM at 5 tons/ha, followed by the control plots which had aflatoxin B_1 in excess of 600 µg/kg. The high aflatoxin levels from plots with a low rate of FYM came from seeds that were not treated with fungicides or bio agents. Concomitantly, there were high incidences of *A. flavus* and *A. parasiticus* isolates from these samples that might have accounted for high aflatoxin concentrations. None of the aflatoxin G types were detected in the present samples in spite of isolation of *Aspergillus* spp. were employed. All plots subjected to *T. harzianum* as a seed treatment singly and in combinations with different levels of FYM applications were devoid of aflatoxin contaminations in the samples. Among plots subjected to T. viride singly and in

Table 2. Farm yard manure, fungicide and *Trichoderma* species, treatment effects against *Aspergillus* species seed invasions in log CFU/g seed in Babile district, eastern Ethiopia in 2014 and 2015 main cropping seasons.

Treatment	2014				2015			
	A. flavus	***A. flavus* S-strain**	***A. parasiticus***	***A. niger***	***A. flavus***	***A. flavus* S-strain**	***A. parasiticus***	***A. niger***
Negative control	1.08	0.00	0.00	0.00	1.63	0.30	1.00	0.30
FYM 2.5 tons/ha	0.78	0.00	0.00	0.00	0.85	0.00	0.30	0.00
FYM 5 tons/ha	1.30	0.30	0.48	0.00	0.90	0.30	0.48	0.00
FYM 7.5 tons/ha	0.69	0.00	0.00	0.00	0.69	0.00	0.30	0.00
Carbendazim 2 g/kg	0.00	0.00	0.00	0.00	0.69	0.00	0.30	0.00
Mancozeb 3 g/kg	0.60	0.00	0.00	0.00	1.36	0.00	1.30	0.00
T. harzianum 5 g/kg	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30
T. viride 5 g/kg	0.48	0.30	0.00	0.00	0.78	0.30	0.00	0.00
FYM 2.5 tons/ha + Carbendazim 2 g/kg	0.69	0.00	0.00	0.48	0.69	0.00	0.00	0.69
FYM 2.5 tons/ha + Mancozeb 3 g/kg	0.00	0.00	0.00	0.30	0.00	0.00	0.00	0.30
FYM 2.5 tons/ha + T. harzianum 5 g/kg	0.69	0.48	0.00	0.78	0.60	0.00	0.00	0.30
FYM 2.5 tons/ha + T. viride 5 g/kg	0.00	0.00	0.00	0.00	0.48	0.00	0.60	0.00
FYM 5 tons/ha + Carbendazim 2 g/kg	0.60	0.00	0.00	0.00	0.69	0.00	0.30	0.00
FYM 5 tons/ha + Mancozeb 3 g/kg	0.85	0.00	0.00	0.00	0.60	0.00	0.00	0.30
FYM 5 tons/ha + T. harzianum 5 g/kg	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
FYM 5 tons/ha + T. viride 5 g/kg	0.30	0.30	0.00	0.30	0.60	0.30	0.48	0.60
FYM 7.5 tons/ha + Carbendazim 2 g/kg	0.85	0.30	0.00	0.00	1.18	0.30	0.00	0.00
FYM 7.5 tons/ha + Mancozeb 3 g/kg	0.30	0.30	0.00	0.00	0.95	0.30	0.48	0.00
FYM 7.5 tons/ha + T. harzianum 5 g/kg	0.00	0.48	0.00	0.00	0.48	0.48	0.30	0.00
FYM 7.5 tons/ha + T. viride 5 g/kg	0.48	0.00	0.00	0.30	0.78	0.00	0.30	0.69

combinations, only samples from plots treated with FYM 7.5 tons/ha + *T. viride* 5 g/kg had aflatoxin B_1 and B_2 that were 99% less aflatoxin contamination than plots with the highest aflatoxin levels.

In the 2015 cropping season, samples (n=60), 63.3% (n= 38) were found to be contaminated by aflatoxins while the remaining 36.7% (n= 22) were apparently free from aflatoxin (Table 3). The highest aflatoxin level of B_1 (5704.4 µg/kg) was detected from the control plots and the lowest B_1 (2.5 µg/kg) from plots treated with FYM at 2.5 tons/ha + *T. harzianum* at 5 g/kg. Unlike the 2014 cropping season samples, aflatoxin G types were detected in plots sown with seeds treated with mancozeb 3 g/kg, the same plot had aflatoxin B_1 (588.0 µg/kg). Perhaps *A. parasiticus* and/or *A. flavus* isolated from these samples had the capability to produce B and G type aflatoxins and apparently, mancozeb could not inhibit those isolates.

In both cropping seasons, plots treated with *T. harzianum* at 5 g/kg seed and FYM 5 tons/ha + *T. harzianum* at 5 g/kg were not contaminated by aflatoxins. In the later year (2015), plots that received FYM 2.5 tons/ha + *T. harzianum* 5 g/kg decreased both aflatoxins B_1 and B_2 by 99.9% as compared to the untreated control plots. In 2014, apart from control plots, only samples from plots treated with FYM 5 tons/ha were positive for aflatoxin with maximum aflatoxin B_1. In the latter year (2015), all single treatment applications were positive for aflatoxins except plots sown with *T. harzianum* 5 g/kg seed. Among plots treated with sole FYM in 2015 cropping season, maximum (9.0 µg/kg) aflatoxin B_1 concentration was estimated in

Table 3. Aflatoxin concentration (in µg/kg seed) in groundnut samples (N=120, n=60 per season) in Babile district, eastern Ethiopia, in the 2014 and 2015 main cropping seasons.

Treatment	2014				2015			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Negative control	651.3	71.0	nd	nd	5704.4	2219.0	nd	nd
FYM 2.5 tons/ha	nd	nd	nd	nd	9.0	1.0	nd	nd
FYM 5 tons/ha	1340.6	76.5	nd	nd	6.5	nd	nd	nd
FYM 7.5 tons/ha	nd	nd	nd	nd	4.2	0.1	nd	nd
Carbendazim 2 g/kg	nd	nd	nd	nd	3.8	0.1	nd	nd
Mancozeb 3 g/kg	nd	nd	nd	nd	588.0	50.1	205.2	24.0
T. harzianum 5 g/kg	nd	nd	nd	nd	nd	nd	nd	nd
T. viride 5 g/kg	nd	nd	nd	nd	50.0	2.3	nd	nd
FYM 2.5 tons/ha + Carbendazim 2 g/kg	nd	nd	nd	nd	6.6	0.3	nd	nd
FYM 2.5 tons/ha + Mancozeb 3 g/kg	10.3	1.4	nd	nd	nd	nd	nd	nd
FYM 2.5 tons/ha + *T. harzianum* 5 g/kg	nd	nd	nd	nd	2.5	0.1	nd	nd
FYM 2.5 tons/ha + *T. viride* 5 g/kg	nd	nd	nd	nd	16.2	3.4	nd	nd
FYM 5 tons/ha + Carbendazim 2 g/kg	nd	nd	nd	nd	6.1	0.5	nd	nd
FYM 5 tons/ha + Mancozeb 3 g/kg	27.6	4.0	nd	nd	3.8	2.7	nd	nd
FYM 5 tons/ha + *T. harzianum* 5 g/kg	nd	nd	nd	nd	nd	nd	nd	nd
FYM 5 tons/ha + *T. viride* 5 g/kg	nd	nd	nd	nd	76.0	9.0	nd	nd
FYM 7.5 tons/ha + Carbendazim 2 g/kg	269.5	13.9	nd	nd	306.8	11.1	nd	nd
FYM 7.5 tons/ha + Mancozeb 3 g/kg	nd	nd	nd	nd	22.4	1.3	nd	nd
FYM 7.5 tons/ha + *T. harzianum* 5 g/kg	nd	nd	nd	nd	15.0	1.2	nd	nd
FYM 7.5 tons/ha + *T. viride* 5 g/kg	6.2	0.5	nd	nd	96.0	5.7	nd	nd

[a]nd: Aflatoxin not detected.

samples taken from plots treated with FYM 2.5 tons/ha, whereas plots that received FYM 7.5 tons/ha had aflatoxin B_1 (4.2 µg/kg), which revealed that the highest rate of FYM application significantly decreased aflatoxin contaminations and vice versa.

Effects of treatments on yield and yield components of groundnut

Yield and yield components varied across treatments but the variation was not significant in most cases regardless of the experimental year (Table 4). In the 2014 experiment, the highest mean of SCE (90.0 plants/plot) recorded from plots treated with FYM 2.5 tons/ha + mancozeb at 3 g/kg, FYM 2.5 tons/ha + *T. viride* at 5 g/kg, FYM 5 tons/ha + *T. viride* 5 g/kg, and FYM 7.5 tons/ha + *T. harzianum* 5 g kg^{-1}. On the other hand, control plots had the lowest number of plants (74.0 plants/plot). The highest mean SCH (33.7 plants/plot) was obtained from plots treated with FYM 5 tons/ha and FYM 7.5 tons/ha + *T. harzianum* 5 g/kg. The highest means PY (670.9 kg/ha), NSP (1.8), and the second highest mean SY (601.0 kg/ha) and NPP (34.9) were harvested from plots treated with FYM at 7.5 tons/ha + mancozeb 3 g/kg. These results were achieved due to the integration of soil amendment with a high amount of FYM and seed treatment with fungicides.

In the 2015 season, 10 different treatments led to the highest (90 plants/plot) SCE (Table 4), the same levels of performances. The lowest (80.3 plants/ plot) SCE was recorded from untreated plots and plots treated with FYM at 5 tons/ha + *T. harzianum* 5 g/kg. In case of SCH, the highest mean (47.7 plants/plot) was obtained from plots treated with FYM at 7.5 tons/ha. The highest PY and SY (1901.5 and 1281.5 kg/ha, respectively) were obtained from pots treated with *T. harzianum* at 5 g/kg. The highest NPP (27.9) was recorded from plots treated with FYM at 5 tons/ha + *T. harzianum* 5 g/kg and the lowest (20.0) was from the control plots.

Days to 50% emergence in experimental years, viz. 2014 and 2015, showed non-significant ($p > 0.05$) differences among treatments (Table 5). Days to 50% flowering showed slight differences among treatments and all plots attained flowering with the mean intervals of 37.0 to 46.0 days. Days to 95% physiological maturity exhibited significant differences among treatments from 146.0 to 152.0 days. Evidently, Tegene et al. (2013) reported an unnamed local variety reaching physiological maturity from 148 to 154 days in eastern Ethiopia.

Pearson correlation coefficients evaluated among the agronomic and yield parameters suggested differential relationships (Table 6). In the 2014 experiment, SY showed highly significant and positive ($r=0.42$, $P= 0.01$)

Table 4. Farm yard manure, fungicide and *Trichoderma* species, treatment effects on yield and yield components of groundnut under field condition in Babile, eastern Ethiopia in the 2014 and 2015 main cropping seasons.

Treatment	SCE	SCH	NPP	PY	NSP	SHP	SY	HSW
				2014				
Negative control	74.0^{b}	27.0^{abcdef}	20.5^{cde}	295.9^{de}	1.7^{a}	64.0^{cde}	258.3^{bc}	40.6^{b}
FYM 2.5 tons/ha	89.0^{a}	25.7^{abcdef}	28.7^{abc}	574.6^{abc}	1.8^{a}	61.8^{e}	599.5^{ab}	39.5^{b}
FYM 5 tons/ha	78.7^{ab}	33.7^{a}	26.5^{abcd}	454.6^{abcde}	1.7^{a}	68.1^{abcd}	420.3^{abc}	40.3^{b}
FYM 7.5 tons/ha	85.7^{ab}	32.0^{abc}	27.3^{abcd}	656.1^{ab}	1.7^{a}	70.2^{ab}	646.9^{a}	40.9^{b}
Carbendazim 2 g/kg	78.7^{ab}	27.0^{abcdef}	28.1^{abc}	529.4^{abcde}	1.6^{a}	67.8^{abcde}	516.0^{abc}	41.5^{b}
Mancozeb 3 g/kg	84.7^{ab}	31.7^{abcd}	26.3^{abcd}	560.9^{abcd}	1.7^{a}	69.6^{abc}	491.7^{abc}	40.6^{b}
T. harzianum 5 g/kg	86.0^{ab}	31.3^{abcd}	26.2^{bcd}	369.7^{bcde}	1.6^{a}	65.3^{abcde}	332.8^{abc}	42.0^{ab}
T. viride 5 g/kg	82.3^{ab}	28.0^{abcdef}	25.9^{bcd}	340.0^{bcde}	1.7^{a}	65.5^{abcde}	290.7^{bc}	39.4^{b}
FYM 2.5 tons/ha + Carbendazim 2 g/kg	89.7^{a}	23.7^{def}	37.5^{a}	585.5^{ab}	1.7^{a}	65.8^{abcde}	531.9^{abc}	54.0^{a}
FYM 2.5 tons/ha + Mancozeb 3 g/kg	90.0^{a}	26.3^{abcdef}	23.3^{cde}	360.2^{bcde}	1.7^{a}	66.0^{abcde}	274.3^{bc}	42.8^{ab}
FYM 2.5 tons/ha + *T. harzianum* 5 g/kg	87.7^{ab}	27.0^{abcdef}	28.1^{abc}	305.3^{cde}	1.6^{a}	64.6^{bcde}	336.3^{abc}	40.0^{b}
FYM 2.5 tons/ha + *T. viride* 5 g/kg	90.0^{a}	28.8^{abcde}	25.4^{abcd}	256.4^{e}	1.7^{a}	63.6^{ed}	220.6^{c}	39.4^{b}
FYM 5 tons/ha + Carbendazim 2 g/kg	89.0^{a}	24.7^{cdef}	21.7^{cde}	261.3^{e}	1.7^{a}	64.1^{cde}	220.7^{c}	42.0^{ab}
FYM 5 tons/ha + Mancozeb 3 g/kg	85.3^{ab}	25.3^{bdef}	27.7^{abcd}	525.2^{abcde}	1.7^{a}	63.7^{cde}	495.2^{abc}	40.2^{b}
FYM 5 tons/ha + *T. harzianum* 5 g/kg	81.0^{ab}	28.3^{abcdef}	21.8^{cde}	360.6^{bcde}	1.7^{a}	65.5^{abcde}	317.4^{bc}	39.2^{b}
FYM 5 tons/ha + *T. viride* 5 g/kg	90.0^{a}	27.0^{abcdef}	23.3^{cde}	279.0^{e}	1.7^{a}	65.0^{bcde}	271.9^{bc}	38.9^{b}
FYM 7.5 tons/ha + Carbendazim 2 g/kg	89.0^{a}	29.0^{abcde}	19.5^{cde}	470.9^{abcde}	1.7^{a}	67.3^{abcde}	444.0^{abc}	41.3^{b}
FYM 7.5 tons/ha + Mancozeb 3 g/kg	88.3^{a}	29.0^{abcde}	34.9^{ab}	670.9^{a}	1.8^{a}	66.7^{abcde}	601.0^{ab}	42.0^{ab}
FYM 7.5 tons/ha + *T. harzianum* 5 g/kg	90.0^{a}	33.7^{a}	20.5^{cde}	372.2^{bcde}	1.7^{a}	63.0^{ed}	362.6^{abc}	42.1^{ab}
FYM 7.5 tons/ha + *T. viride* 5 g/kg	86.0^{ab}	22.3^{ef}	21.0^{cde}	291.9^{de}	1.8^{a}	71.0^{a}	220.8^{c}	39.4^{b}
LSD (0.05)	13.7	8.1	11.3	245	0.2	5.9	337.3	12.3
CV (%)	9.7	17.6	27	39	7.4	5.5	51	18
				2015				
Negative control	80.3^{b}	40.3^{a}	20.0^{a}	1389.2^{a}	1.7^{cd}	63.3^{b}	803.6^{b}	54.0^{bc}
FYM 2.5 tons/ha	90.0^{a}	46.3^{a}	24.1^{a}	1500.6^{a}	1.7^{cd}	64.0^{ab}	991.6^{ab}	52.0^{c}
FYM 5 tons/ha	88.0^{ab}	39.7^{a}	25.5^{a}	1500.6^{a}	1.8^{abcd}	67.1^{ab}	1055.1^{ab}	55.4^{bc}
FYM 7.5 tons/ha	90.0^{a}	47.7^{a}	23.0^{a}	1462.0^{a}	1.7^{cd}	69.9^{ab}	1032.7^{ab}	61.1^{ab}
Carbendazim 2 g/kg	81.0^{b}	41.0^{a}	24.2^{a}	1528.6^{a}	1.7^{cd}	69.2^{ab}	1104.2^{ab}	54.3^{bc}
Mancozeb 3 g/kg	90.0^{a}	45.0^{a}	23.0^{a}	1597.7^{a}	1.9^{abc}	69.4^{ab}	966.4^{ab}	55.0^{bc}
T. harzianum 5 g/kg	90.0^{a}	42.7^{a}	25.2^{a}	1901.5^{a}	2.0^{ab}	68.0^{ab}	1281.5^{a}	56.8^{bc}
T. viride 5 g/kg	85.3^{ab}	42.7^{a}	25.6^{a}	1720.8^{a}	1.8^{abcd}	71.0^{a}	965.2^{ab}	56.0^{bc}
FYM 2.5 tons/ha + Carbendazim 2 g/kg	87.0^{ab}	42.7^{a}	25.3^{a}	1528.8^{a}	1.9^{abc}	69.5^{ab}	931.1^{ab}	51.4^{c}
FYM 2.5 tons/ha + Mancozeb 3 g/kg	90.0^{a}	42.0^{a}	26.3^{a}	1623.3^{a}	2.0^{ab}	63.0^{b}	1126.0^{ab}	54.2^{bc}
FYM 2.5 tons/ha + *T. harzianum* 5 g/kg	88.0^{ab}	43.7^{a}	22.3^{a}	1405.6^{a}	1.8^{abcd}	68.0^{ab}	1186.2^{ab}	57.6^{abc}
FYM 2.5 tons/ha + *T. viride* 5 g/kg	90.0^{a}	42.0^{a}	21.3^{a}	1632.4^{a}	1.8^{bcd}	64.0^{ab}	1035.2^{ab}	56.6^{bc}
FYM 5 tons/ha + Carbendazim 2 g/kg	90.0^{a}	40.7^{a}	24.3^{a}	1579.0^{a}	1.9^{abc}	63.3^{b}	988.3^{ab}	53.5^{bc}
FYM 5 tons/ha + Mancozeb 3 g/kg	90.0^{a}	42.3^{a}	24.2^{a}	1678.1^{a}	1.7^{cd}	65.6^{ab}	1045.2^{ab}	54.3^{bc}
FYM 5 tons/ha + *T. harzianum* 5 g/kg	80.3^{b}	39.7^{a}	27.9^{a}	1532.4^{a}	1.8^{bcd}	64.9^{ab}	1170.4^{ab}	55.4^{bc}
FYM 5 tons/ha + *T. viride* 5 g/kg	84.0^{ab}	42.7^{a}	25.3^{a}	1523.0^{a}	2.1^{a}	65.6^{ab}	1028.9^{ab}	55.4^{bc}
FYM 7.5 tons/ha + Carbendazim 2 g/kg	90.0^{a}	44.3^{a}	22.4^{a}	1576.8^{a}	1.8^{bcd}	67.1^{ab}	1010.2^{ab}	65.8^{a}
FYM 7.5 tons/ha + Mancozeb 3 g/kg	90.0^{a}	42.7^{a}	21.0^{a}	1439.0^{a}	1.9^{abc}	65.2^{ab}	1018.7^{ab}	52.6^{bc}
FYM 7.5 tons/ha + *T. harzianum* 5 g/kg	88.0^{ab}	42.3^{a}	23.0^{a}	1621.2^{a}	1.7^{cd}	69.2^{ab}	1089.6^{ab}	56.9^{bc}
FYM 7.5 tons/ha + *T. viride* 5 g/kg	88.0^{ab}	45.0^{a}	21.1^{a}	1532.7^{a}	1.7^{cd}	63.0^{b}	1020.7^{ab}	53.9^{bc}
LSD (0.05)	8.8	9.8	10.6	567.2	0.3	7.5	385.9	8.7
CV (%)	6.1	14.0	27.4	22.0	10.6	6.8	22.5	9.5

[a]SCE: Stand count at emergence (in number); [b]SCH: stand count at harvest (in number); [c]HSW: hundred seed weight (g); [d]SHP: shelling percentage (%); [e]PY: pod yield (kg ha^{-1}); [f]SY: seed yield (kg ha^{-1}); [g]NPP: number of pods per plant (mean values); [h]NSP: number of seeds per pod (mean values). [i]LSD: Least significant difference; [j]CV: coefficient of variation. [k]Means with the same letters are not significantly different.

Table 5. Farm yard manure, fungicide and *Trichoderma* species effects of treatments on days to 50% emergence and flowering, and 95% physiological maturity of groundnut in Babile, eastern Ethiopia in 2014 and 2015 main cropping seasons.

Treatment	2014			2015		
	D50%E	D50%F	D95%M	D50%E	D50%F	D95%M
Negative control	20.3[a]	37.0[c]	149.7[ab]	20.3[a]	40.3[ab]	149.7[abc]
FYM 2.5 tons/ha	18.0[a]	41.0[abc]	149.3[abc]	18.0[a]	43.3[ab]	149.3[abc]
FYM 5 tons/ha	19.7[a]	38.0[bc]	146.0[e]	19.7[a]	41.7[ab]	148.3[abc]
FYM 7.5 tons/ha	19.0[a]	37.3[bc]	149.7[ab]	19.0[a]	42.7[ab]	149.0[abc]
Carbendazim 2 g/kg	19.0[a]	44.0[ab]	149.0[abcd]	19.0[a]	43.3[ab]	148.7[abc]
Mancozeb 3 g/kg	19.0[a]	41.0[abc]	148.7[abcd]	19.0[a]	45.0[a]	148.0[bc]
T. harzianum 5 g/kg	18.7[a]	44.0[ab]	148.7[abcd]	18.7[a]	39.3[b]	149.3[abc]
T. viride 5 g/kg	19.0[a]	42.3[abc]	149.3[abc]	19.0[a]	40.7[ab]	148.3[abc]
FYM 2.5 tons/ha + Carbendazim 2 g/kg	19.0[a]	46.0[a]	150.7[a]	19.0[a]	41.0[ab]	150.0[abc]
FYM 2.5 tons/ha + Mancozeb 3 g/kg	19.3[a]	43.7[abc]	149.7[ab]	19.3[a]	40.3[ab]	150.0[abc]
FYM 2.5 tons/ha + *T. harzianum* 5 g/kg	18.0[a]	42.0[abc]	147.3[cde]	18.0[a]	38.7[b]	149.3[abc]
FYM 2.5 tons/ha + *T. viride* 5 g/kg	18.3[a]	42.0[abc]	149.3[abc]	18.3[a]	41.3[ab]	147.3[c]
FYM 5 tons/ha + Carbendazim 2 g/kg	18.7[a]	40.3[abc]	149.3[abc]	18.7[a]	43.0[ab]	150.0[abc]
FYM 5 tons/ha + Mancozeb 3 g/kg	18.3[a]	41.3[abc]	147.0[ed]	18.3[a]	40.3[ab]	149.7[abc]
FYM 5 tons/ha + *T. harzianum* 5 g/kg	19.0[a]	42.7[abc]	148.7[abcd]	19.0[a]	43.3[ab]	148.3[abc]
FYM 5 tons/ha + *T. viride* 5 g/kg	19.7[a]	40.3[abc]	148.3[bcd]	19.7[a]	41.7[ab]	152.0[a]
FYM 7.5 tons/ha + Carbendazim 2 g/kg	18.0[a]	43.7[abc]	149.7[ab]	18.0[a]	39.3[b]	148.0[bc]
FYM 7.5 tons/ha + Mancozeb 3 g/kg	19.3[a]	40.3[abc]	148.7[abcd]	19.3[a]	39.7[b]	151.7[ab]
FYM 7.5 tons/ha + *T. harzianum* 5 g/kg	20.0[a]	43.0[abc]	150.0[ab]	20.0[a]	41.7[ab]	150.0[abc]
FYM 7.5 tons/ha + *T. viride* 5 g/kg	19.0[a]	43.0[abc]	149.3[abc]	19.0[a]	38.7[b]	150.0[abc]
LSD (0.05)	2.8	7.0	2.2	2.8	5.1	3.7
CV (%)	8.8	10.1	0.9	8.8	7.4	1.5

[a]D50%E: Days to 50% emergence; [b]D50%F: days to 50% flowering; [c]D95%M: days to 95% maturity; [d]LSD: least significant difference; [e]CV: coefficient of variation.

correlation with NPP; correspondingly PY had significant positive (r= 0.94 and 0.40, P= 0.01) correlations with SY and NPP. Similarly, Vaithiyaungan et al. (2010) found that SY of groundnut had highly significant and positive association with NPP and harvest index. In the 2015, PY (r= 0.43, P= 0.01) and SHP (r= 0.41, P= 0.01) were significantly and positively correlated with SY.

DISCUSSION

Plots sown with groundnut seed treatments with fungicides (carbendazim and mancozeb) or biocontrol (*T. harzianum* and *T. viride*) had a minimum invasion by *Aspergillus* species compared with sole FYM application. Fungicide seed treatment is effective in reducing losses caused by *A. niger* in crops like groundnut which are vulnerable at the seedling stage (Manju et al., 2017), thereby enhancing the crop resistance and contributing for harvesting of healthy seed. Biocontrol agents of *T. viride* and *T. harzianum* have also shown benefits in managing the collar rot of groundnut (Pratibha et al., 2012; Gangwar et al., 2014). The effectiveness of integrated soil organic amendments of FYM with biocontrol agent (*T. harzianum*) and fungicide (carbendazim) against *Aspergillus* invasion was evaluated (Manju et al., 2017). Presently, the samples obtained from plots treated with FYM 2.5 tons/ha integrated with mancozeb 3 g/kg and *T. viride* 5 g/kg and FYM 5 tons/ha + *T. harzianum* 5 g/kg had less invasion by *Aspergillus* spp. Particularly, the samples from plots treated with *Trichoderma* spp. in single and integrated with FYM showed lower invasions of *Aspergillus* spp. Mohamed (2015) reported that groundnut seed treated with bioagents produced healthier and higher yielding plants than control. *Trichoderma* spp. are widely used in agriculture as biopesticides, bioprotectants, biostimulants, and biofertilizers on a wide variety of plants (Ranasingh et al., 2006).

The plots treated with carbendazim at 2 g/kg seed had less invasion when compared with samples from control plots. These results are in agreement with the observation of Reddy et al. (2008) who reported a significant reduction in the growth of *Aspergillus* spp. and aflatoxin

Table 6. Simple Pearson correlation coefficients of growth parameters, yield and yield components of groundnut in Babile, eastern Ethiopia, in 2014 and 2015 main cropping seasons.

Correlation	2014								2015							
	SCE	SCH	HSW	SHP	PY	SY	NPP	NSP	SCE	SCH	HSW	SHP	PY	SY	NPP	NSP
SCE		-0.22	0.08	0.16	0.12	0.04	-0.17	0.24		0.22	0.03	0.07	0.47**	0.06	-0.01	-0.04
SCH			-0.11	0.13	0.01	0.02	0.02	-0.07			-0.04	-0.01	-0.04	-0.07	-0.28*	-0.11
HSW				0.11	0.05	-0.03	0.11	0.12				0.23	0.24	0.27*	-0.25	-0.11
SHP					0.22	0.12	-0.06	0.14					0.31*	0.41**	-0.02	0.02
PY						0.94**	0.40**	0.24						0.43**	0.08	-0.09
SY							0.42**	0.15							0.09	-0.05
NPP								-0.03								0.09
NSP																

[a]*Correlation is significant at the 0.05 level (2-tailed); [b]**Correlation is significant at the 0.01 level (2-tailed).

contaminations of rice that received seed treatment with carbendazim at 3 g/kg. In contrast, the prevalence of *A. flavus* and *A. parasiticus* was high in samples collected from plots sown with mancozeb treatment at 3 g/kg seed. However, Getnet et al. (2013) reported that mancozeb 3 g/kg and carbendazim 2 g/kg seed treatment were effective in suppression of seed invasion by *A. flavus* and *A. parasiticus* than *A. niger*, where as plots sown with carbendazim 2 g/kg or mancozeb 3 g/kg seed treatment were found to be free of *A. niger* in our study. Treatments were also evaluated on the aflatoxin contamination reduction. In the first year (2014) experiment, 90% samples were negative for aflatoxins, while 10% had detectable aflatoxin concentrations, of that 33% had aflatoxin B_1 below 10 µg/kg. Studies have shown that aflatoxin B_1 concentration in food above 10 µg/kg is considered hazardous and a threat to food security (Lewis et al., 2005). Plots treated with FYM at 5 tons/ha had aflatoxin B_1 of 1340.6 µg/kg, and the same plots were infected with *A. flavus* and *A. parasiticus* which might have been responsible for the accumulated aflatoxin level. It has been reported that high aflatoxin concentration accumulates in the soils and if microorganisms do not rapidly degrade it, it will be absorbed by the roots of crops in the subsequent years and translocated to other plant parts like seeds and fruits (Mertz et al., 1980). Samples harvested from the plots planted with *T. harzianum* alone and integrated with different rates of FYM had aflatoxin below the detectable levels. Perhaps the antagonistic effect of *T. harzianum* coated to planted seeds likely inhibited the *Aspergillus* spp. and inhibited aflatoxin production in those plots. The current finding is in agreement with the report of Benizri et al. (2001).

In the second year (2015), the highest aflatoxin B_1 (5704.4 µg/kg) and B_2 (2219.0 µg/kg) were detected in samples harvested from the control plots. Recently Mohammed et al. (2016) detected aflatoxin B_1 2526.3 µg/kg in groundnut seed collected from growers' storages. These results indicated that, the groundnut seed produced in eastern Ethiopia was badly contaminated with aflatoxin. However, of the contaminated samples, 55% had aflatoxin B_1 below acceptable levels (10 µg/kg) set by some countries like China which is 20 µg/kg (Xiaoxia et al., 2015); 15 µg/kg for Taiwan (Chen et al., 2013), and 10 µg/kg for Korea and Uganda (Kaaya et al., 2006; Ee et al., 2007). This indicates that pre-harvest management decrease aflatoxin accumulation. Plots subjected to FYM 7.5 tons/ha + *T. harzianum* 5 g/kg had aflatoxin B_1 levels of 15.0 µg/kg. Plots sown with seeds treated with *T. harzianum* 5 g/kg, FYM 2.5 tons/ha + mancozeb 3 g/kg, and FYM 5 tons/ha + *T. harzianum* 5 g/kg showed no aflatoxin contamination. The findings affirmed that the use of *T. harzianum* as seed treatment or in combinations with FYM significantly reduced seed invasion by *Aspergillus* spp. and aflatoxins contamination. Choudhary (1992) also reported that, *Trichoderma* spp. inhibited aflatoxin B_1 by 73.5% and G_1 by 100% produced by *A. flavus*. Soil amendment with FYM significantly reduced aflatoxin contamination. Waliyar et al. (2007) reported that treatment with FYM 2.5 tons/ha reduced aflatoxin contamination by 42%. However, in the current study the same rate reduced aflatoxin concentration by 99.8% in 2015 cropping season and 100% in 2014.

The proportion of aflatoxin contaminated samples and concentrations were higher in 2015 cropping season than samples harvested in 2014. The results were in agreement with the prevalence of *Aspergillus* spp. Perhaps, the amount of rainfall received in 2015 cropping season (446.2 mm) contributed to higher aflatoxin levels compared to the preceding year (2014) which received 596 mm of rainfall during the growing season. In fact, drought stress is the principal factor contributing to *Aspergillus* spp. occurrences and aflatoxin production under field conditions. Researchers (Waliyar et al., 2003; Craufurd et al., 2006) investigated drought stress as the main factors that predispose seeds to aflatoxigenic fungi and aflatoxin contamination in the field. Groundnut seed samples harvested from rainfed conditions under moisture stress had a maximum (10,240 µg/kg) concentration of

aflatoxin, while traces amount were detected in samples from well irrigated plots (Mehan et al., 1988). In the current finding, the highest total aflatoxin (B_1+B_2+G_1+G_2= 7, 923.4 µg/kg) was observed in the control plot samples in the 2015 crop season, while 1,417.1 µg/kg aflatoxin levels were detected in the 2014 samples. This is in agreement with the variable amount of rainfall received during 2014 and 2015 crop seasons.

The PY and SY obtained from plots treated with FYM 2.5 tons/ha + *T. viride* 5 g/kg indicated that low rate of FYM application resulted in low yields, while the highest corresponding mean PY and SY were recorded from plots subjected to treatment with FYM at 7.5 tons/ha + mancozeb 3 g/kg and FYM at 7.5 tons/ha, respectively. These findings are in accordance with the result of Waliyar et al. (2006), who obtained an increase in yield with FYM supplement at different cropping stages contributing to increased groundnut yield. The estimated national and global yield of groundnut in 2014/2015 was about 1600 kg/ha (FAOSTAT, 2014; CSA, 2014, 2015). However, in the current study the highest yield of 1901.5 kg/ha was achieved in plots treated with *T. harzianum* 5 g/kg which was higher than the yields reported at the national level and globally. This could be due to growth and yield enhancement in addition to the antagonistic effects of bioagents against groundnut pathogens. CSA (2009) reported that, groundnut yields with good management practiced could rise to 3 tons/ha, supporting the present finding. The increase in yield is also attributed to increased vigor of healthy plants through growth regulators produced by *Trichoderma* spp., which improved plant photosynthesis according to Govindappa et al. (2011). Albeit, *Trichoderma* spp. seed treatments contribution for photosynthesis improvement should not be ruled out in the current study.

Application of FYM at 10 to 15 tons/ha has been reported to increase the pod and haulm yields and improved the yield parameters compared to inorganic fertilizers (Subrahmaniyan et al., 2000). In the current study, plots treated with FYM at 7.5 tons/ha had the highest average mean of PY (1029.2 kg/ha) and SY (844.4 kg/ha). This indicated that the highest rate of FYM applications could produce better yields. The present finding is in agreement with the report of Lokanath (2010), who confirmed heavy application of FYM at 75 tons/ha producing higher dry pod yield (3510 kg/ha) of groundnut in better moisture conditions. Higher soil moisture in organically amended plots generally leads to poor aeration and decreased activity of soil microorganisms and affects the nutrient availability in the soils and subsequently reduces the yield components of the crop (Lokanath, 2010). In the current study, the amount of rainfall received during 2014 experimental year was 596 mm and PY of 596.4 kg/ha was obtained, while in 2015 when the rainfall was less (446.2 mm) a PY of 1462.0 kg/ha was obtained from plots treated with FYM 7.5 tons/ha. Generally, the important yields of PY and SY were higher in 2015 than 2014 experimental year.

Among the agronomic data, the required days for 50% emergence, 50% flowering, and 95% maturity were 18.0 to 20.3, 37 to 46.0, and 146.0 to 152.0 days, respectively in the current study. Likewise, the same variety "*Oldhale*" used by Bethlehem (2011) reported that, it took 17, 36.4, and 140.3, days to attain 50% emergence, 50% flowering and 95% physiological maturity, respectively, in Babile district. MoARD (2009) reported that an improved variety, Fetene, took 27 to 35 to attain 50% flowering and 115.9 days to 95% physiological maturity; while "*Oldhale*" took relatively longer time to reach the specified stages. However, Jeyaramraja and Fantahun (2014) reported that Tole 2 variety required 157 days to reach 95% physiological maturity, much later than "*Oldhale*". In case of the Pearson correlation coefficient, the experiment indicates that PY and SY contributed a great deal to the total yields of groundnut. Bethlehem (2011) reported that NPP was significantly and positively correlated with SY. NPP and NSP were significant components of groundnut yield and could be utilized as yield indicators. Parameswarappa et al. (2008) revealed that NPPs are an important yield component of the crop.

This study has demonstrated the effects of FYM on the management of pre-harvest aflatoxins in groundnuts. As a conclusion, in the eastern part of Ethiopia farmers apply FYM for maintaining soil fertility in some cash crops but the awareness of its effects on plant disease management is quite less. However, in the current study, FYM effects on the groundnut yields, fungal invasions and aflatoxin contamination reductions in single and integrated with seed treatment produced important information for small-scale growers. Therefore, the practice of using FYM for soil fertility improvement by farmers in Eastern Ethiopia should be encouraged. The study also affirmed that, *T. harzianum* as seed treatment had tremendous effects in reducing *Aspergillus* spp. invasion and subsequent aflatoxin contaminations in groundnut. Therefore, the future research should be focused on either development of resistant varieties against aflatoxin contaminations, looking for non-toxigenic biocontrol strains, or alternatively developments of simple and easy formulations of *T. harzianum* for small-scale farmers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Physiological characterization of NADP-malic enzyme activity under 2,4-D toxicity in an aquatic fern, *Azolla pinnata* R.Br.

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This work proposes NADP-ME activity as bio-indices for herbicidal sensitivity in *Azolla pinnata* R.Br. under 2,4-D toxicity. *Azolla* fronds were illuminated under saturated photosynthetic flux and bicarbonate induction. There was significant variation in the NADP-ME activity under light and dark conditions. This increased more when varying concentrations of 2,4-D were used for the up regulation of enzyme activity especially under illumination. There was evident modulation through activators (citrate and succinate) and inhibitors (pyruvate and oxaloacetate) under light and dark conditions at 2,4-D concentration. This occurs at the same time in overriding the herbicidal stress linked to the adjustment of the cellular redox of regulatory sites using dithiothreitol. This shows that *Azolla* can quench 2,4-D, which suggests NADP-ME as a biomarker through its sensitivity and mode of activities under xenobiotic contaminated soil.

Key words: NADP-malic enzyme, aquatic fern, herbicide, modulators, malate.

INTRODUCTION

There are few key regulatory enzymes in carbon reduction pathways of plants where both concentrations of cellular metabolites as well as physico-chemical factors are responsible for regulation. NADP-malic enzyme (NADP-ME: L-Malate; NADP-oxido-reductase [oxaloacetate de-carboxylating]) is a oxidative decarboxylating enzyme regardless of C_3 and C_4 plants that regenerate CO_2 at the expense of NADP (Xu, et al., 2013). NADP-ME [EC-1.1.1.40] is most crucial for photosynthesis in respect to re-fixation of CO_2 from oxidative decarboxylation of malate with generation of $NAD(P)H+H^+$. Moreover, malate, the substrate for NADP-ME, is the influencing factor of photosynthetic flux through ratio of $NADP^+$ to $NAD(P)H+H^+$. The redox potential of tissue by $NADP^+$: $NAD(P)H+H^+$ is now a crucial factor for different abiotic stress responses, and

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thus NADP-ME serves as an index for stress sensitivity (Wang et al., 2017). The activity of NADP-ME is under the control of few cellular inputs like high pH (≥8.0), high CO_2, malate and HCO_3^- concentration in the chloroplast. This is the chloroplastic light regulatory protein indexed in oxidative stress (Turkan et al., 2018). Any fluctuation of abiotic inputs may be a factor to determine the optimum activity of this enzyme. Environmental stresses changing the cellular redox may be marked by NADP-ME activities and its deviation from controlled conditions. Thus, NADP-ME also serves as a redox indicator under changing condition of photo oxidation, photorespiratory condition of high light intensities and low CO_2:O_2 ratio (Kustka et al., 2014).

Photo oxidation is accomplished by few abiotic stressors in the form of toxic chemical residues, predominantly sourced by industrial effluents. Those chemicals may be artificial and hazardous to environment distributed through biological system. Overall, these are called xenobiotics, and in the context of pollutants, dioxins and polychlorinated biphenyl derivatives are emphasized (Neilson, 2017). Out of the agricultural pollutants, herbicides are the predominant ones included with xenobiotics. Non-target plant species are more vulnerable to herbicides through the residual effect of 2,4-D in soil. Herbicide has been more frequently reported in different strategies to de-contaminate the soil after weeding (Gill and Garg, 2014). A systematic herbicide is also realised through its residual in soil after its absorption by roots and its downstream effects in plants (Foy, 2018). Waste water, effluents from industry and run-off from agricultural fallows, and herbicidal residues contaminate surface water. Effluents from waste water treatment plant containing 80% herbicides could greatly contaminate surface water (Tournebize et al., 2017). In aquatic ecosystem marshy plants predominantly interact with herbicidal toxins in relation to their hyperaccumulation and sustenance. Regarding herbicidal stress, photo oxidation is the determining factor for sustainability in plants (Anjum et al., 2011). With this, the relevance of NADP-ME may be questioned as a regulatory index for tolerance to herbicides. Few aquatic macrophytes including some non-flowering plants like *Salvinia*, *Marsilea*, *and Azolla* were also reported for their hyper-accumulating nature (Das et al., 2013; Dhir, 2018). *Azolla*, an aquatic fern, is more established with its inherent nitrogen fixing ability to contribute to soil fertility. In addition, it can greatly prevent herbicides from contaminating water bodies. Based on this, we hypothesize that *Azolla* may be a bio-indicator species for detecting 2,4-D toxicity through the modulation of NADP-ME activities. Therefore, NADP-ME is used as the bio-index for the changes that occur in cellular redox under 2,4-D toxicity. Thus, in the present experiment, the NADP-ME activity under 2,4-D toxicity is discussed in *Azolla* and its modulation under light and darkness that may open up the regulatory mode of the enzyme.

MATERIALS AND METHODS

Plant material and experimental conditions

Azolla pinnata R.Br., a free floating aquatic fern, was collected from the fresh water pond in the university campus for the present experiment. Initially, plants were transferred to cemented tank filled with tap water for further use. Thereafter, plants were transferred to a nutrient solution containing ¼th MS media for acclimatization (Murashige and Skoog, 1962). Thereafter, the plants were supplemented with varying concentrations of 2,4-D: 100, 250, 500 and 1000 µM for 7 days in growth chamber at 35 ± 1°C, 85% RH and 14/10 h (L/D).

Biochemical analysis

After 7 days of treatment the plants were harvested and separated into root and fronds and stored at -80˚C. Later, they were used for different biochemical assays.

NADP-ME activity

NADP-ME activity was determined according to Drincovich et al. (2001). Discs of *Azolla* fronds were incubated on 20 ml of 2 mM $NaHCO_3^-$ in Petri dish. They were illuminated within saturated light at an intensity of 900 –1000 $\mu Em^{-2}s^{-1}$. After 30 min of illumination and dark incubation, the tissues were extracted in a prechilled mortar and pestle with 1.5 ml of extraction buffer containing 100 mM Tris HCl (pH=7.5), 10 mM $MgCl_2$, 2 mM EDTA, 10 mM PMSF, 10% (v/v) glycerol, 20% PVP and 10 mM β-ME. The extract was centrifuged at 15000×g for 15 min. The supernatant was used for further enzymatic assay. The enzyme activity was assayed spectrophotometrically at 340 nm by measuring the NADPH produced with 3 ml of assay mixture containing 100 mM Tris-HCl (pH=7.5), 10 mM $MgCl_2$, 0.5 mM $NADP^+$ and enzyme extract. The reaction started after adding 0.01 mM malate (limiting) and 4 mM malate (saturating) concentrations.

Influence of NADP-ME activity under different modulators:

For the determination of regulatory activities of enzyme both from illuminated and dark-adapted fronds under 0.01 and 4 mM malate concentrations, two activators: 2 mM citrate and 2 mM succinate and two inhibitors: 10 mM pyruvate and 0.5 mM oxaloacetate were used for the present experiment, according to Murmu et al. (2003). The extracted and purified protein was used to determine the effect of activators and inhibitors. 3 ml of assay mixture was read at 340 nm to assay the enzyme activity as described earlier.

Regulation of light induction by DTT

The light induced activation of many photosynthetic enzymes is modulated by the reduction of dithiols on the active site residue. In the present experiment the enzyme extracted from the illuminated and dark incubated fronds were assayed by adding and withdrawing 10 mM DTT along with other assay buffer mixture as mentioned above. The reaction started after adding 0.01 mM and 4 mM malate concentrations. The enzyme activity was assayed spectrophotometrically at 340 nm by measuring the NADPH produced, according to Murmu et al. (2003).

Statistical analysis

All the data were recorded with three replications (n=3) and data

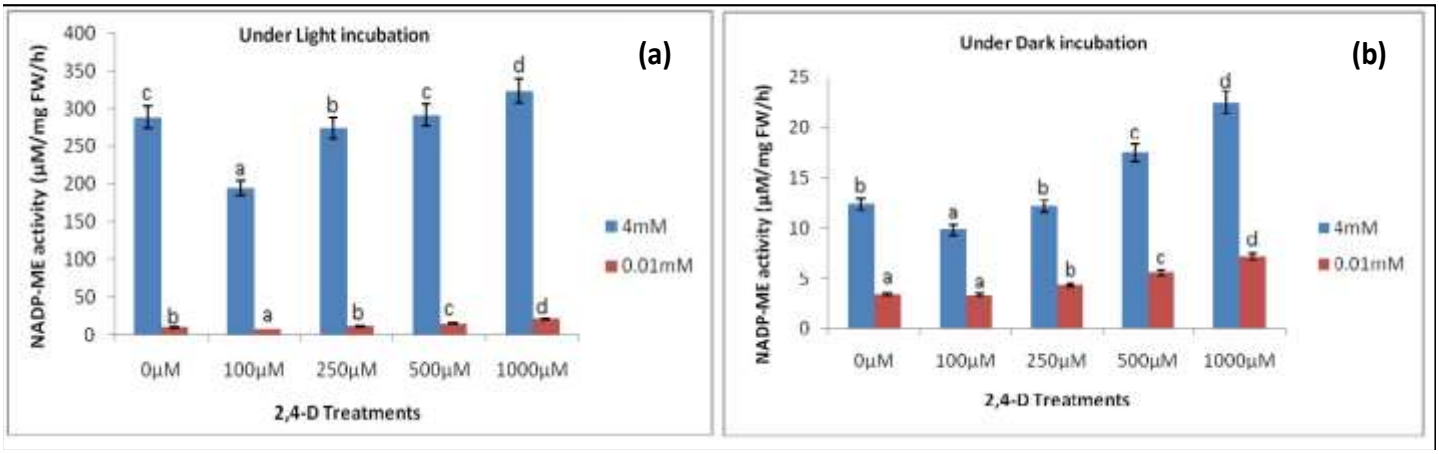

Figure 1. NADP-ME activity in *A. pinnata* R.Br. fronds at 2,4-D concentrations (0, 100, 250, 500 and 1000 µM) incubated under light **(a)** and dark **(b)** conditions and saturated (4 mM) and limited (0.01 mM) malate concentrations. The data were plotted from means of three replications (n=3) ±SE. The different alphabets on each bar show the level of significance (p≤0.05) between the treatments, according to Duncan's test.

were expressed as mean ± SE. The statistical analysis was performed by one-way (ANOVA) followed by least significance difference (LSD) test taking p≤0.05 levels of significance (Gomez and Gomez, 1984). Windows Microsoft Excel 2007 software was employed for computation and data analysis.

RESULTS

Response pattern of light and darkness on NADP-ME activities

The optimum activities of NADP-ME in frond tissues of *A. pinnata* R.Br. were standardised upon illumination around the saturation of PAR (900 – 1000 µE $m^{-2}s^{-1}$) in a solution of 2 mM HCO_3^-. From the two sets of experiment, the observations were made either in illuminated or non-illuminated conditions. The assay mixture was pre-incubated in alkaline medium (pH=7.5- 8.0) under saturated 4 mM and limiting 0.01 mM malate. From both the tissue extract containing partially purified protein recorded an optimum activity (µM/mg FW/h) while being incubated under illumination and darkness for 30 min. The dark-adapted tissues were almost non-responding under the concentrations of 2,4-D; however, a significant discrepancy was recorded compared to that under light. This undoubtedly proves the efficacy of light under constant HCO_3^- concentration as against dark. The optimum activity was not significantly (p≤0.05) different as compared to control as the plants proceed through 2,4-D concentrations (except at initial concentration). In comparison to control (0 µM 2,4-D), the values of activity were curtailed by 33.46 and 47.8% under 100 µM 2,4-D at 0.01 mM and 4 mM malate respectively. However, the peak activity was recorded at 1000 µM 2,4-D by 1.12 and 2.104 fold as compared to control at 4 mM and 0.01 mM malate concentration respectively (Figure 1).

NADP-ME activity and its kinetics under chemical modulators

NADP-ME increases both at transcripts and protein level when tissues vary with irradiance gradient. The pre-incubation in HCO_3^- solution for the optimum enzyme activity was earlier standardised at 2 mM, according to Raghavendra (2000). Contextually, the light activation of NADP-ME through HCO_3^- conceives the effects of activators/ inhibitors as recorded in the present experiment. Likewise, two sets of activators (2 mM citrate and 2 mM succinate) and inhibitors (10 mM pyruvate and 0.5 mM oxaloacetate) were applied. For convenience of experimental analysis, the maximum concentration of herbicide (1000 µM) as tolerated by *Azolla* plants was the standard compared to control (0µM) when it interacts with both the effectors (activators and inhibitors) under malate concentrations.

Regardless of malate concentrations, both activators and inhibitors recorded significant variations in NADP-ME activity under light and dark conditions. For limiting malate concentrations (0.01 mM), the effects of activators (citrate and succinate) were maximised more in light than in the dark. Thus, plants from herbicide treatment were more induced by citrate in light by 1.41 and 1.12 fold over darkness at 0 µM and 1000 µM respectively. Another activator, succinate had similar trend but not significant (p≤0.05) due to the effect of light and darkness. In brief, the activators had modulated the NADP-ME activities under herbicidal toxicity through the effects of light and darkness at the ratios of 1.18 and 1.03 (Figure 3). For inhibitory activity pyruvate and oxaloacetate were chosen; they had similar trend in their mode under light and darkness, but no significant variation. Still, the reduction of activity under herbicide was 42.11% less in darkness than light. The maximum inhibition was done by

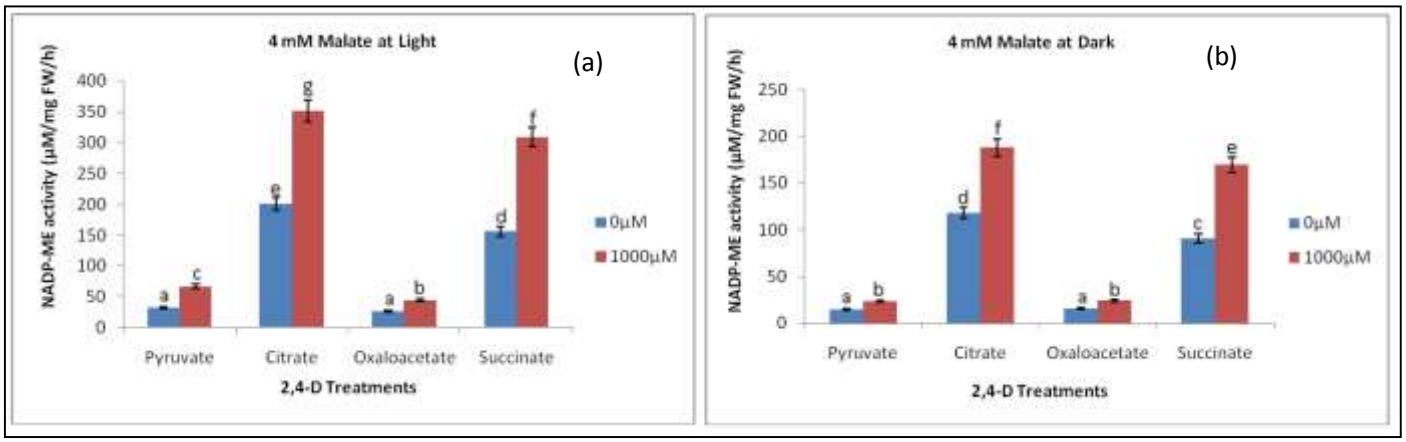

Figure 2. NADP-ME activity in *A. pinnata* R.Br. fronds at control (0 µM) and 1000 µM 2,4-D concentration, incubated under light **(a)** and dark **(b)** conditions and saturated (4 mM) malate concentrations using two activators: 2 mM citrate and 2 mM succinate and two inhibitors: 10mM pyruvate and 0.5 mM oxaloacetate. The data were plotted from means of three replications (n=3) ±SE. The different alphabets on each bar show the level of significance (p≤0.05) between treatments, according to Duncan's test.

oxaloacetate at 42.6% under dark condition compared to light under same malate concentration.

On the contrary, at saturated malate concentration the effect of irradiance is more pronounced for both activators and inhibitors. On average, citrate and succinate increased the enzyme activity by 1.18 fold and 1.03 fold more under light than darkness (Figure 2). Pyruvate and oxaloacetate also inhibited the activities when compared at 1000 µM 2,4-D toxicity against light and dark treatments. Pyruvate minimized more the activity by 42.11% more in darkness than under light (significant at p≤0.05); whereas, for oxaloacetate it was 42.59% in the same condition.

Reversal of light induced changes by redox system

Most of the Calvin cycle enzymes are photo regulatory and are based on changes of redox through di-sulphide (S-S) residues of the amino acids in active site. NADP-ME is no exception and could be modulated by light induced reduction of S-S. In the present experiment, this was also monitored with the partial purified protein incubated with 10 mM DTT under 0.01 and 4 mM malate when frond was immerged with 2 mM HCO_3^- in darkness/illumination. DTT had a significant role regardless of malate concentrations under darkness/light through herbicidal concentration. Thus, the L/D value under DTT presence recorded 0.59 at 0 µM 2,4-D which changes to 0.52 at 1000 µM 2,4-D when assay was done with limiting malate concentration. On the other hand, at saturated malate concentration the presence of DTT recorded a L/D value of 0.71 at 0 µM 2,4-D against 1000 µM 2,4-D by 0.74 L/D value. In the withdrawal or absence and presence of DTT under both concentrations of malate, the changes of L/D values are 1.38, 1.21 and 0.99, 0.92 under 0µM and 1000 µM 2,4-D respectively (Figure 4).

DISCUSSION

This paper highlights photosynthetic metabolism and its monitoring in herbicidal toxicity as biomarker. From the introduction it appears that photosynthetic enzyme, NADP-ME activity may be taken as redox indicator (Yamori et al., 2018). Therefore, under changing 2,4-D concentrations that disturb the cellular redox may accumulate some bio molecules inducing oxidative stress (Biswal et al., 2011). In our earlier studies *Azolla* has been reported as a hyperaccumulater of 2,4-D and can quench residual herbicides by antioxidation (De et al., 2017). Herbicide, in different forms, is a serious bottleneck to fertility and disturbs soil micro flora (Wani et al., 2012). In our earlier reports 2,4-D can be hyperaccumulated and transformed chemically into less toxic moiety in *Azolla* species (De et al., 2017). Therefore, the responses of this species to gene expression would manifest the possible bio indication of herbicidal toxicity. Thus, an initial change of NADP-ME under 2,4-D treatments regardless of light/darkness, activator/inhibitor may suggest its cellular responses under changing redox as said earlier to C_4 and C_3 flowering species. Now in the present experiment, the enzyme activity was evaluated with different conditions and chemicals in *Azolla*. 2 mM HCO_3^- concentration may induce NADP-ME for optimum activity at 4mM malate than 0.01mM. High concentration of CO_2 in the bundle sheath is required to continue the photosynthesis even with minimum CO_2 compensation point (Bellasio and Griffiths, 2013). This establishes it as an attributing factor for light activation of NADP-ME and that is also under

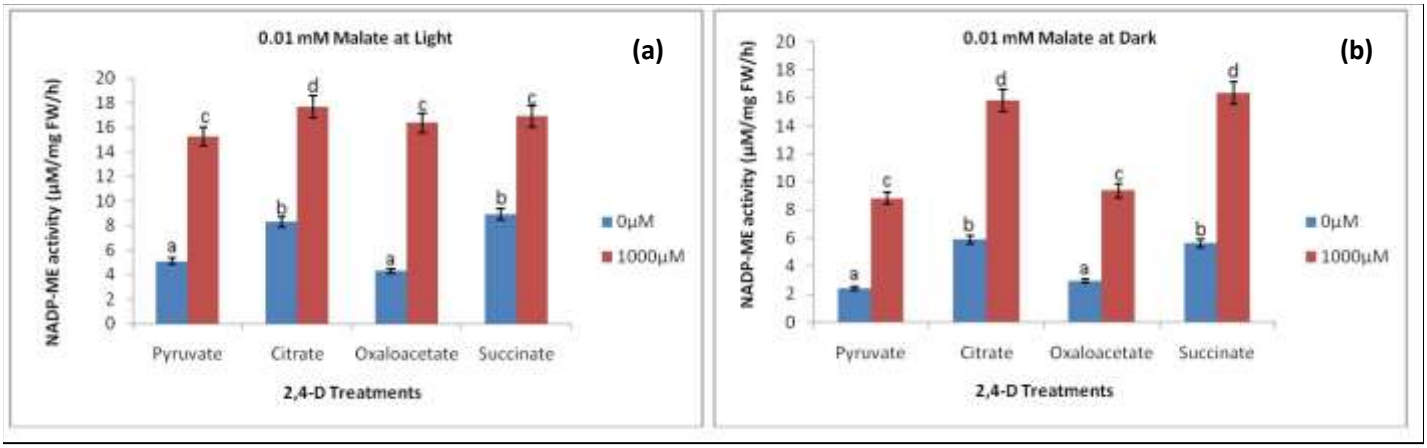

Figure 3. NADP-ME activity in *A. pinnata* R.Br. fronds at control (0 µM) and 1000 µM 2,4-D concentration, incubated under light **(a)** and dark **(b)** conditions and limited (0.01 mM) malate concentrations using two activators: 2mM citrate and 2mM succinate and two inhibitors: 10mM pyruvate and 0.5mM oxaloacetate. The data were plotted from means of three replications (n=3) ±SE. The different alphabets on each bar show the level of significance (p≤0.05) between the treatments, according to Duncan's test.

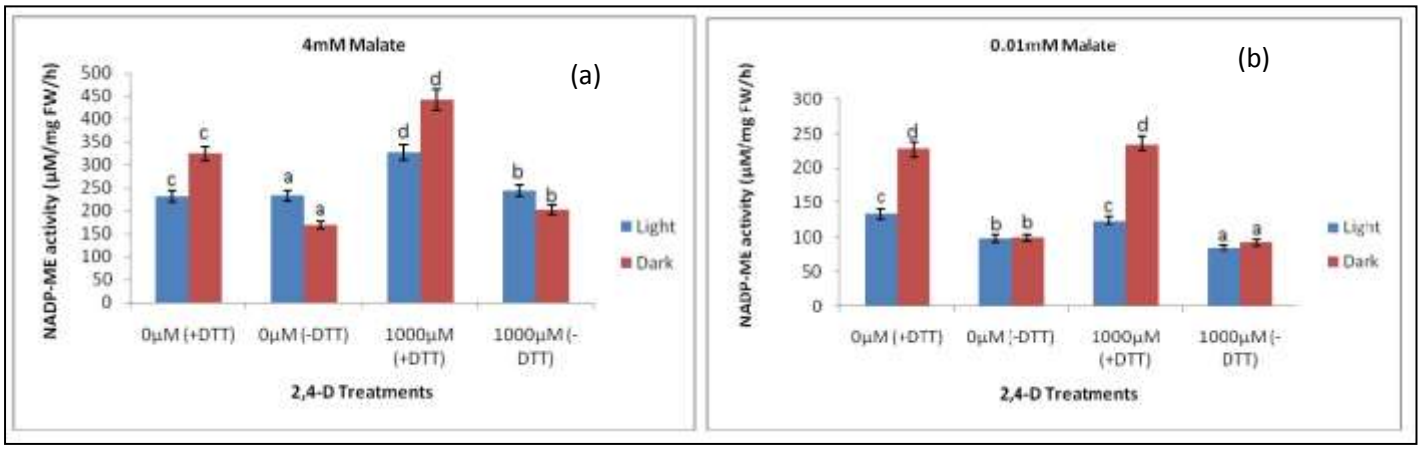

Figure 4. NADP-ME activity in *A. pinnata* R.Br. fronds at control (0 µM) and 1000 µM 2,4-D concentration with/without DTT , incubated under light and dark conditions and saturated (4 mM) **(a)** and limited (0.01 mM) **(b)** malate concentrations. The data were plotted from means of three replications (n=3) ±SE. The different alphabets on each bar show the level of significance (p≤0.05) between treatments, according to Duncan's test.

2,4-D toxicity in *Azolla*. In comparison to control (0 µM 2,4-D), the activity was improved based on the ongoing concentrations of herbicide. This may be attributed to high CO_2 concentration in the tissues. It also supports the tolerance of 2,4-D in *Azolla* with relation to sustained photosynthesis through malate metabolism. In *Azolla,* a C_3 species under the hyperaccumulation of 2,4-D, the photosynthetic activity remains less altered through anaplerotic reactions of organic acids. Malate and its inter-conversion is the most possible source of CO_2 in other downstream reactions (Ewe et al., 2018). Expectedly, in *Azolla* any loss of metabolites would be compensated by an over-riding malate accumulation even under excessive accumulation of herbicides. In higher plants during C_4 photosynthesis, decarboxylation reactions lead to high concentration of CO_2 for RuBP-case activity and thereby photo respiratory loss is minimized (MacAdam and Nelson, 2017). It is assumed that *Azolla* under varying 2,4-D concentrations is compensated for photo respiratory CO_2 loss with NADP-ME overexpresion. 2,4-D can alter the carboxylating machinery and release CO_2 inducing photo respiratory flux.

The photo-activation may not be simple under the influence of light and darkness; yet the responses of *Azolla* to pyruvate (as inhibitor) to decrease NADP-ME activity is interesting. The presence of pyruvate increasing the activity (under illumination) favours the

binding to regulatory domain of the enzyme. Similarly, it happens also for oxaloacetate and malate concentration depending on the feedback inhibition of decarboxylation reactions (Tissier, 2018). Any way, 2,4-D may induce more pyruvate conversion into oxaloacetate releasing CO_2 and thus inhibition results. The supply of CO_2 in the form of HCO_3^- could be another source to enrich the CO_2 over its limiting concentration (Sage, 2013). The influence of HCO_3^- may also be useful to adjust the higher pH of mesophyll cell to tolerate stress. Through photo respiratory flux, especially under high irradiance (near the saturated photo oxidative condition) cytosol becomes alkaline by decarboxylation reactions. Tolerance to herbicides and other toxic compounds may also be attributed by a higher pH of salt accumulation (Powles, 2018). This possibly could be adjusted, however, partially through over -expression of NADP-ME activity by decarboxylating reactions releasing CO_2. Therefore, NADP-ME could be hypothesized as an effective biomarker (yet to be established by isozymes from different plant tissues). Studies have shown that higher angiospermic plants are more flexible to reach optimum activities at 2 mM HCO_3^-, while *Azolla* needs a higher concentration. This is because it can tolerate higher pH and stress (Pandey et al., 2013). *Azolla*, a C3 plant is prone to photo respiratory flux and thus involves in photo-oxidative damages of bio molecules. Despite this, few aquatic plants are still tolerant to photo-oxidative condition due to metal acquisition and being hyperaccumulators of xenobiotics (Gautam, 2017).

The light regulation of Calvin cycle enzymes in higher plants, *Azolla* also added some information regarding its modulation under 2,4-D concentration. From our results the reduction by DTT to the oxidized thiol (-S-S-) at regulatory site might lead to activation of enzyme. Under dark condition the inactivation is realized with redox mediated thiol oxidation of Calvin cycle enzymes (Mock and Dietz, 2016). It is interesting to note that *Azolla* at the pattern of 2,4-D inactivation, particularly, at higher concentrations was quite consistent and compatible with those of angiospermic species. This predicts that the target sites are the thiol ligands for 2,4-D mediated de-activation of NADP-ME (under substrate saturation condition). Therefore, *Azolla* which has already been recognised as hyper-accumulator species for toxic metalloids might be a good indicator for such herbicidal toxicity. It is quite clear that the regulatory mechanism for Calvin cycle enzymes by changes of thiol-redox is compatible irrespective of plant species (including non-flowering species). This also attenuates a change in cellular redox in down regulation of photosynthetic activity through decarboxylation reactions as illustrated with NADP-ME. So, cellular redox with its oxidised/reduced state would complement the NADP-ME activity *in-vitro* as a possible bio-marker under herbicidal stress. *Azolla*, an aquatic fern has served these findings of such conception in the present experiment.

Conclusion

Azolla culture system undoubtedly has an input in nitrogen supplementation in field. Herbicidal toxicity as residual effect in soil is amplified through food chain. In bioremediation mode, a plant species would be an option to quench that much herbicide from soil as well as to detect its presence. The latter is recognized as bio monitoring with some precise cellular responses. NADP-ME activity is evident as a biomarker for herbicidal toxicity expressed through *Azolla* system. This has given more details with its regulation under environmental factors like light and darkness. Along with practical implication, the *Azolla* plants have also revealed the insights for cellular regulation of NADP-ME through biochemical modulation. Therefore, this study would be thought-prone to predict NADP-ME as a reliable bio-indices for 2,4-D toxicity in soil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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ABBREVIATIONS

2,4-D, 2,4-Dichloro phenoxy-acetic acid; **NADP-ME,** NADP linked Malic Enzyme; **ROS,** Reactive Oxygen Species; **RH,** Relative Humidity; **MS Media,** Murashige and Skoog media; **$MgCl_2$,** Magnesium Chloride; **PMSF,** Phenylmethylsulfonyl fluoride; **EDTA,** Ethylene diamine tetraacetic acid; **DTT,** Di-thio threitol.

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Full Length Research Paper

Effects of seedling age and rates of phosphorus fertilizer on growth and yield performance of onion (*Allium cepa* L.) under irrigation at Alage, Central Rift Valley of Ethiopia

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Onion is an important cultivated crop as a condiment as well as a source of income for many farmers in Ethiopia. However, the productivity of the crop is much lower due to different problems. A field experiment was conducted to determine the effect of seedling age and phosphorus rate on growth and yield performance of onion at Alage, Central Rift Valley of Ethiopia, during 2016/2017 season. The treatments comprised three seedling ages (6, 7 and 8 weeks of seedling age) and four phosphorus rates (0, 46, 92 and 138 kg P_2O_5ha^{-1}). The experiment was laid out in randomized complete block design (RCBD) with four replications. The result showed that seedling age and phosphorus rate significantly affected plant height, leaf length, days to maturity, fresh bulb weight, bulb dry matter fraction, bulb length, marketable bulb yield, total bulb yield, and harvest index. Among these parameters, marketable bulb yield, total bulb yield and harvest index were also significantly affected by the interaction of seedling age and phosphorus rate. On the other hand, leaf number per plant, bulb diameter, total biomass yield and unmarketable bulb yield were only influenced by the effect of phosphorus rates. In this study, transplanting at 8 weeks of seedling age fertilized with 138 kg P_2O_5 ha^{-1} recorded the highest total bulb yield (50.6 t ha^{-1}) and marketable bulb yield (48.33 t ha^{-1}), but no significant difference was shown with that obtained at 92 kg P_2O_5 ha^{-1} with the same seedling age. Treatment combinations of seedling age at 6 weeks and no P (control) produced the lowest amounts of total bulb yield (24.27 t ha^{-1}) and marketable bulb yield (21.63 t ha^{-1}). The partial budget analysis revealed that the highest net benefit with low cost of production was obtained in response to the application of 92 kg P_2O_5 ha^{-1} and the transplanting age of 8 weeks. The marginal rate of return for this treatment was 5657% which is found to be economically feasible for producing onion in the study area.

Key words: Onion, seedling age, phosphorus, bulb yield, partial budget analysis.

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important monocotyledonous, herbaceous, cross-pollinated and cool season vegetable crops, which belongs to the genus *Allium* and the family Alliaceae (Griffiths et al., 2002). It is important in the daily diets of human's worldwide and in Ethiopia as well (MoARD, 2006).

Onion is grown from seed, transplants or sets for use as both green onions and dry onion (Decoteau, 2000). It is bulbous, biennial herb which gives off a distinctive and pungent odor when the tissues are crushed (Ray and

Yadav, 2005). It is a popular vegetable in Ethiopia and produced in many home gardens and commercially in different parts of the country. Its production is rapidly increasing both under rain-fed and irrigation conditions (Fekadu and Dandena, 2006). The mature bulb contains some starch, appreciable quantities of sugars, some protein, and vitamins A, B and C (Jilani et al., 2010). In the study area, onion crop plays an important role in contributing to the household food security. In addition to the nutritional value, these crops generate employment opportunities for the poor households in the district area. The crop is also one of the most important cash crops that generate income for the farmers in the study area. Ethiopia has diversified agro-climatic conditions which is suitable for the production of a broad range of vegetables and flowers, and allows successful production of onion crop (FAO, 1998). Onion is important cash crop for the farmers in Ethiopia; and hence the crop is produced in different parts of the country for local consumption and for export market. However, due to the various constraints the average productivity of onion in Ethiopia is 10.7 t ha^{-1} (CSA, 2013) which is far below the world average of 19.5 t ha^{-1} (FAO, 2011).

Application of fertilizer is important for production of onion. Among the nutrients, nitrogen and phosphorus play the most important role for vegetative growth and root development of the crop which ultimately helps in increasing bulb size and total yield (Rai, 1981). Onions are generally established either by direct seeding or by bare root transplants. Compared to direct seeding, transplanted onions provide an immediate and complete stand. The effect of transplant age on yield is an issue often broached by growers of horticultural and agronomic crops in an effort to maximize production potential (Leskovau and Vavrina, 1999). Although production of onion variety is “Bombay Red”, expanding information on optimal phosphorus fertilizer application rate and proper age of seedling is scanty. Systematic study on fertilization to improve the growth and yield of bulb is lacking. Onion producers in the area use blanket recommendation of phosphorus fertilizer which was recommended at country level. As Ethiopia has a diversified agro-ecology and soil conditions, site specific fertilizer recommendation is needed to improve productivity and production of onion. On the other hand, farmers transplant onion based on their own judgment on the age/size of seedlings which critically influence the productivity and quality of the bulb. Both late and early age transplanting of seedlings may have significant influence on survival and growth performance of onion. In view of the existing problem, this study was proposed with the objective to determine the effect of seedling age and rates of phosphorus fertilizer on growth and bulb yield of onion.

MATERIALS AND METHODS

Description of the study area

The study was conducted at Alage Agricultural Technical and Vocational Education and Training (ATVET) College during the 2016/2017 dry season under irrigation. The site is located at 217 km south of Addis Ababa city and 32 km west of Bulbula town in the vicinity of Abidjata and Shalla lakes. It is situated between 7° 65’ N latitude and 38° 56’ E longitude and at an altitude of 1600 m above sea level in the agro ecology of dry plateau of the southern part of the Ethiopian rift valley system. High amount of rainfall is received in the month of July and August. While the mean annual rainfall is 800 mm, the annual mean minimum and maximum temperatures are 11 and 29°C, respectively (Agerie and Afework, 2013).

Experimental Materials

Plant Material

The plant material for this study was Bombay Red variety of onion. The variety is widely accepted by farmers for its early maturity and higher bulb yield in the study area. It was released by Melkasa Agricultural Research Center (MARC) in 1980. It is well adapted to areas of 700 to 2000 m above sea level (EARO, 2004). It is one of the most commonly and widely used improved variety in Central Rift Valley of Ethiopia and particularly at Alage.

Fertilizer

The sources of the fertilizers were urea (46% N) and Triple Super Phosphate (TSP) (46% P_2O_5) for supplying nitrogen and phosphorus, respectively.

Treatments and experimental design

The experiment comprised of 3 × 4 factorial combinations involving age of seedling and varying rates of phosphorus (P). Three age of seedlings (6, 7 and 8 week of seedling age) and four varying rates of P (0, 46, 92, and 138 kg P_2O_5 ha^{-1}) were laid out in randomized complete block design (RCBD) with four replications. Each treatment combination was assigned randomly to the experimental units within a block. Double row planting was done on ridges of about 20 cm height adopting recommended spacing of 40 cm between water furrows, 20 cm between rows on the ridge and 10 cm between plants within the row. There were 48 plots corresponding to the 12 treatment combinations with four replications. The unit plot size of the experiment was 2.0 m × 2.0 m (4 m^2). The blocks were separated by a distance of 1.0 m whereas the space between each plot within a block was 50 cm. In each plot, there were 10 rows, and in each row there were 20 plants. Totally, there were 200 plants per plot. The outer two rows at both sides of the plot and two plants at both ends of the rows were considered as border plants. The plants in the six central rows were

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used as net plot area to determine yield per plot and other parameters.

Agronomic practices and treatment applications

Raising onion seedlings

Seedlings of 'Bombay Red' onion variety were raised in a nursery at Alage ATVET College demonstration site on sunken beds with size of 1 m × 10 m. The seed of 'Bombay Red' was obtained from Melkasa Agricultural Research Center, Horticulture Division. After four sunken nursery beds were prepared, seeds were sown on December 15, 2016. The soil was cultivated to a fine tilth before sowing the onion seed. The seed was drilled in well pulverized sunken bed in rows 10 cm apart and lightly covered with soil in the required seedling age. Mulching with grasses was done until seedlings emerged. The mulch was removed after seedlings fully emerged (2 to 3 cm from the soil). All important cultural practices such as application of fertilizer (urea and DAP), watering, weed, diseases (Ridomil) and insect pest (Selecron) control activities, respectively were undertaken based on the recommendations made for the onion crop.

Management of experimental field

Before transplanting seedlings, the experimental field was ploughed and leveled by tractor; ridges and plots were made manually. Large clods were broken down in order to bring the land to a fine tilth, and then a total of 48 plots based on recommended size were prepared in which 12 plots were allocated in each of the four replications. Moreover, the required numbers of ridges and rows were marked in each plot. The seedlings were grown in the nursery with careful management and strict follow up until seedlings reached to the required stage as per the treatments. Seedlings were hardened before transplanting to the main field to enable them withstands the field conditions. The experiment was conducted under furrow irrigation method, which is the most commonly used irrigation system in the study area. A three to four days irrigation interval was maintained for the first four weeks. Thereafter, irrigation was applied at 7 days interval until 15 days remaining to harvest, when irrigation was stopped completely (EARO, 2004).

As per the recommendation made for the onion in the study area, half 50 kg ha^{-1} dose of N was applied uniformly to all plots during transplanting. The remaining half 50 kg ha^{-1} dose of nitrogen rates was side-dressed 45 days after transplanting for all plots (EARO, 2004; SARC, 2008; Anisuzzaman et al., 2009). Phosphorus (TSP) was applied as a single application as per specified rates at the time of transplanting based on the treatments. Weeding was done with hand hoe and by hand-pulling whenever necessary throughout the experimental period to keep the crop free from weeds, for better soil aeration and to break the crust. For the control of disease (purple blotch) and insect pest (onion thrips), the fungicides, Mancozeb 80 WP (3 kg ha^{-1}) plus Ridomil (3.5 kg ha^{-1}) and the insecticide, Selecron 720 EC (0.5 L ha^{-1}) were used, respectively. All other agronomic practices were applied uniformly for all the plots as per the recommendation made for the crop (EARO, 2004).

Soil sampling and analysis

Pre-planting soil samples were taken randomly in a zigzag fashion from the experimental field at the depth of 0 to 20 cm for determination of physical and chemical properties of the soil. Nine soil samples were collected using an Auger from the whole experimental field and combined to form a composite sample in a bucket. From this mixture, a sample weighing 1 kg was filled into a plastic bag. The soil samples were also analyzed for soil texture, total nitrogen, cation exchange capacity (CEC), exchangeable potassium, organic carbon and available phosphorous. All the analyses were made at Horticoop Ethiopia P.L.C. soil and water analysis laboratory in Debre Ziet.

Data collection

Days to maturity was registered on plot basis. Growth and yield components were recorded from twelve sample plants randomly taken from six central middle rows of each experimental plot. However, all plants in each net plot were harvested to collect data for bulb yield.

Partial budget analysis

Partial budget analysis was conducted to assess the economic feasibility of the treatments. It is a method of organizing experimental data and information about the costs and benefits of various alternative treatments. Partial budget, dominance and marginal analysis were used. The analysis was based on data collected from respective district office of trade and transport, cooperatives and from onion fields. At Alage, the cost of 100 kg phosphorus (TSP) was 1095 birr and onion price of 400 birr per 100 kg was used for the net benefit analysis. A partial budget is a way of calculating the total costs that vary and the net benefits of each treatment (CIMMYT, 1988).

Statistical data analysis

The collected data on various parameters under study were subjected to analysis of variance (ANOVA) using the GLM procedures of Statistical Analysis System (SAS) version 9.2 computer software program (SAS Institute Inc, 2008). Significance of differences between means was expressed using the least significance difference (LSD) test at $P< 0.05$ probability level.

RESULTS AND DISCUSSION

Physico-chemical properties of the experimental soil

The results revealed that the texture of the composite soil sample from the site was silty clay loam. The soil had particle size distribution of 18% sand, 36% silt and 46% clay at the depth of 0 to 20 cm. The pH was slightly alkaline (pH=7.82). Brewster (1994) stated that for higher yield of onion, a pH of 6.0 to 6.8 is ideal. However, the pH of this soil is near optimum for onion crop production, although it is not ideal. According to the rating of Walkley and Black (1954) and Dewis and Freitas (1975), the soil of the study area is medium in organic carbon (2.10%) as well as total nitrogen (0.18%), respectively. The cation exchange capacity (CEC, 31.8 meq/100 g) of the experimental soil is also high according to the rating of Jackson (1975) and low in phosphorus (4.12 mg/kg) according to Olsen et al. (1954).

Phenology and growth parameters

Days to maturity

Days to maturity of onion were significantly ($P < 0.001$)

Table 1. Main effect of seedling age and P fertilizer rates on mean plant height, leaf length, number of leaves per plant, days to physiological maturity, bulb diameter and bulb length.

Treatment	Days to maturity	Plant height (cm)	Leaf length (cm)	Number of leaves/plant	Bulb diameter (cm)	Bulb length (cm)
Seedling age(weeks)						
6	102.06^{a}	55.26^{b}	44.24^{b}	11.97	4.39	4.46^{b}
7	99.00^{b}	57.27^{ab}	45.49^{b}	11.43	4.32	4.52^{b}
8	96.75^{c}	59.30^{a}	47.78^{a}	11.89	4.49	4.98^{a}
LSD(0.05)	1.07	2.041	1.92	-	-	0.40
Significance level	***	**	**	NS	NS	*
P_2O_5 (kg ha^{-1})						
0	101.17^{a}	53.58^{c}	42.00^{c}	10.44^{c}	4.11^{b}	4.10^{c}
46	100.00^{a}	56.17^{b}	44.90^{b}	11.44^{b}	4.33^{ab}	4.41^{bc}
92	98.58^{b}	60.35^{a}	48.87^{a}	12.58^{a}	4.58^{a}	4.75^{b}
138	97.33^{c}	59.00^{a}	47.57^{a}	12.59^{a}	4.59^{a}	5.35^{a}
LSD(0.05)	1.23	2.36	2.22	0.96	0.31	0.46
Significance level	***	***	***	***	**	***
CV (%)	1.49	4.95	9.86	5.82	8.38	11.91

Means followed by the same letters within a column are not significantly different at ($P < 0.05$).

affected by the main effects of seedling age and phosphorus treatments, but not significantly affected by the interaction effects of the treatments.

The present result showed that, seedling age at 6 and 7 weeks took relatively longer days for onion bulb to mature as compared to the 8 weeks of seedling age. Onion plants with 8 weeks of seedling age reached maturity six and three days earlier than 6 and 7 weeks of seedling age, respectively (Table 1). This result is in agreement with that of Kumbhkar et al. (2016) and Bijarniya et al. (2015), who reported that onion seedlings that were transplanted at early stage were delayed to attain maturity. Early maturity of bulbs might be due to vigorous growth of plants because of stored food in older seedlings which results in faster development and earlier maturity of bulb as compared to other young seedlings (Bijarniya et al., 2015). These results are also in accordance with those of Deepika (2013) who reported that plants produced from 30 day old seedlings took more time to mature than those obtained from 40, 50 and 60 day old seedlings.

Days to maturity was also significantly delayed (101 days) in plants from unfertilized plots by phosphorus, while plots received phosphorus at the rate of 138 kg P_2O_5 ha^{-1} matured earliest (97 days) followed by 92 kg ha^{-1} P rate (99 days) (Table 1). However, no significant difference was observed between unfertilized treatments and those received 46 kg P_2O_5 ha^{-1} plots. Generally, plants grown under higher phosphorus rates tended to be early matured. The result indicates that the shortened time required by the plants to reach maturity at higher rates of phosphorus fertilizers might be attributed to the role of P in plants, that it is used in dry matter distribution which facilitates plant development, as a result, early maturity of the plants. The significant function of phosphorus enhances photosynthesis, reproduction, flowering, fruiting, including seed production and maturation of plants (Brady and Weil, 2002). The current observation is in line with Ahn (1993) who indicated that P is concentrated in the fast growing parts of the plant; therefore, it hastens the maturing period of crops.

Plant height

Result from the ANOVA revealed that the main effects of seedling age ($P<0.01$) and phosphorus ($P < 0.001$) significantly influenced plant height of onion. However, the interaction of the two factors did not influence plant height.

There was a significant difference in plant height at maturity among the various weeks of seedling age recorded (Table 1). Numerically, the highest plant height was obtained from the plant when they were transplanted at 8 weeks of seedling age followed by the plants at 7 weeks of seedling age, while the shortest was obtained under 6 weeks of seedling age but statistically similar with 7 weeks of age. In general, the longer plant height at the oldest seedling might be due to more time of completion before transplanting stimulating height of seedlings as compared to the youngest seedling age which resulted in better establishment and vigorous growth of plant but shorter in height. Similarly, Bijarniya et al. (2015) reported that the more plant height at 8 weeks

old seedling stage might be due to their greater stored food present in them as compared to the 6 old ones. This result is also in line with that of Sultana (2015) who found that the maximum plant height was recorded from 7 weeks old seedlings.

Increasing the rate of phosphorus fertilizer from nil up to 92 kg P_2O_5 ha^{-1} resulted in a significant increase in plant height. However, increasing the rate of phosphorus from 92 to 138 kg P_2O_5 ha^{-1} did not change the height of the onion plants. The tallest mean plant heights were attained at 92 kg P_2O_5 ha^{-1}, while the shortest plant height was obtained under the control treatment. The mean height of onion plants grown at the rate of 92 kg P_2O_5 ha^{-1} exceeded the mean height of plants grown at the rates of 46 and 0 kg P_2O_5 ha^{-1} by about 7 and 13% , respectively (Table 1). This might be attributed to the fact that phosphorus enhances plant vigor and strength of the stem of the plant (Bahadur et al., 2002). The result of this study confirms the findings of Ali et al. (2008), Aliyu et al. (2007) and Tibebu et al. (2014) who reported that different phosphorus levels resulted in significantly different plant heights where the tallest plants were observed at higher rates of applied phosphorus, while the shortest plants were from the control plots. The result also agreed with the findings of Lemma and Shimeles (2003) who reported that at 92 kg P_2O_5 ha^{-1} there was better vegetative growth which resulted in increased heights of onion plants. Similarly, Getachew (2014) reported that the height of the plants was increased as the level of phosphorus fertilizer increased from 0 to 115 kg P_2O_5 ha^{-1}, but further increase of fertilizer application, decreased the height of the plants.

Leaf length

Leaf length of onion plants was significantly affected by the main effects of seedling age and phosphorus treatments. However, leaf length was not significantly affected by the interaction effect of those treatments. This study indicated that, the longest leaf was observed when seedlings are transplanted at 8 weeks of age, while numerically the shortest leaf length was obtained under 6 weeks of seedling age (Table 1). The result is supported by observations of Singh and Chaure (1999) who reported that longer leaves were recorded at old seedling age as compared to treatments of the young ones. Muhammad et al. (2017) and Sultana (2015) also reported that the length of leaf was significantly influenced by the different age of seedling of onion.

Application of 138 kg P_2O_5 ha^{-1} gave significantly longer leaf length than the lower doses (0 and 46 kg ha^{-1}), whereas the 92 kg ha^{-1} P treatment is not significantly different from 138 kg P_2O_5 ha^{-1} in leaf length (Table 1). The shortest leaf length was obtained under the control treatments. Also significant difference was observed between the 46 and 92 kg ha^{-1} P rates. In general, leaf length tended to show an increasing trend as phosphorus rate increases. The leaf length response to phosphorus fertilizer rate is in agreement with Fageria (2003) who reported that higher rates of phosphorus resulted in longer leaves of onion. The positive effect of phosphorus on leaf length might be due to the fact that it contains an essential component of nucleic acids, phospholipids, and some amino acids and absorbed phosphorus helped a direct stimulation of cellular activity in roots and leaves (Jawar et al., 2016).

Number of leaves per plant

The main effect of phosphorus fertilizer application significantly ($P < 0.001$) influenced the number of leaves per plant of the onion at physiological maturity. However, neither the main effect of seedling age nor its interaction with phosphorus influenced this parameter of the onion.

The result indicated that, effect of different rates of phosphorus on the number of leaves per plant increased with the increase in phosphorus level. The highest number of leaves was obtained from 138 kg P_2O_5 ha^{-1}, whereas the lowest leaf number was obtained under the control treatment. Increasing the rate of phosphorus from nil to 92 kg P_2O_5 ha^{-1} significantly increased the number of leaves per plant of onion. But, the mean leaf number per plant did not show significant difference with further increase in phosphorus rate from 92 to 138 kg ha^{-1}. Thus, the mean leaf number per plant of onion treated with phosphorus at the rate of 92 kg ha^{-1} exceeded the leaf number per plant of onion treated with nil and 46 kg P_2O_5 ha^{-1} by about 21 and 10%, respectively (Table 1). This might be attributed to the role of P in plants, that it is used in dry matter distribution, which facilitates plant development.

The increase in the number of leaves as a result of the increased rates of phosphorus application may be attributed to the useful role of P for the process of cell division and meristematic growth. Vachhani and Patel (1993) also observed that increased application of phosphorus rate increased the number of leaves per plant. Similarly, Fatma et al. (2012) reported that the higher level of P fertilizer at 103 kg ha^{-1} significantly increased plant height, number of green leaves per plant, bulb and neck dimensions and fresh and dry weights of whole plant and its different organs as compared with the lower level of 69 kg ha^{-1}.

Yield and yield components of onion

Bulb diameter: Phosphorus had significant ($P<0.01$) effect on mean bulb diameter of onion plants. However, the main effect of seedling age as well as its interaction effect with phosphorus did not affect this parameter. Applications of phosphorus at 46, 92 and 138 kg P_2O_5 ha^{-1}

gave similarly larger bulb diameters as compared to the plants without added phosphorus. The increment in bulb diameter due to phosphorus application might be due to the fact that phosphorus improved the carbohydrate content of the plants and it extended root growth, which ultimately increased the blub size (Jawar et al., 2016). Regarding the rates, Shaheen et al. (2007) reported that bulb diameter significantly increased with the increases of phosphorus fertilizer up to 92 kg ha^{-1}. Tibebu et al. (2014) also reported that the highest bulb diameter was obtained from 69 kg P_2O_5 ha^{-1}.

Bulb length: The main effect of seedling age ($P<0.05$) and phosphorus ($P<0.001$) significantly affected mean bulb length of onion plants; however, the interaction effect of seedling age and phosphorus fertilizer did not result significant difference ($P > 0.05$). The result indicated that, at 6 weeks of seedling age, the bulb length was significantly reduced, but no significant difference was observed with 7 weeks of seedling age. Compared with the 6 and 7 weeks of seedling age treatments, 8 weeks of seedling age produced 12 and 10% longer bulbs, respectively. The highest bulb length was observed with transplanting of 8 weeks of seedling age, while numerically the lowest bulb length was recorded in 6 weeks of seedling age (Table 1). This indicated that the 8 weeks of seedling age had maintained longer leaves and greater height which may have helped for more vegetative growth and bulb development and ultimately an increase in length of bulb. This result is in line with that of Singh and Chaure (1999) and Bahadur and Singh (2005) who observed that increase of bulb length at older seedling age as compared to treatments of the young seedling age. Similarly, Sultana (2015) reported that length of bulb increased in 50 days old seedlings, which were strong and larger in size at transplanting stage. In response to rising the rate of phosphorus from nil to 46, 92 and 138 kg P_2O_5 ha^{-1}, bulb length increased significantly. The highest bulb length was observed from 138 kg P_2O_5 ha^{-1} followed by 92 kg P_2O_5 ha^{-1}, while significantly smaller bulb length was obtained under the control treatments (Table 1). The significant increase in bulb length in response to the increment in the rate of phosphorus fertilizer may be linked to the increase in dry matter production and its partitioning to the bulb. The bulb length was higher with the application of phosphorus and this might be due to the fact that the phosphorus improved the carbohydrate content of the plants and it extended root growth, which ultimately helped in the increased length of onion bulb (Khodadadi, 2012).

Fresh bulb weight: The main effect of seedling age ($P<0.01$) and phosphorus fertilizer rate ($P<0.001$) significantly influenced the fresh bulb weight of the onion plants, but not by the interaction of the two main factors. The result revealed that, at 6 weeks of seedling age, the fresh bulb weight was significantly reduced, but no significant difference was observed with 7 weeks of seedling age (Table 2). Compared to the 6 and 7 weeks of seedling age treatments, 8 weeks of seedling age produced 6 and 9% larger bulbs, respectively. The highest fresh bulb weight was recorded from plots that were transplanted at 8 weeks of seedling age, while the least fresh bulb weight was obtained when seedlings were transplanted at 6 weeks of seedling age. It was possible that old seedling age took less time to recover from the transplanting shock in order to get established in the field (Latif et al., 2010). The result is supported by observations of Singh and Chaure (1999) and Mohanty et al. (1990) who reported that increased fresh bulb weight was obtained at old seedling age as compared to treatments of the young ones. When the rate of phosphorus was increased from 0 to 46 and 92 kg P_2O_5 ha^{-1}, there were significant increments in fresh bulb weight. When the rate of fertilizer was increased from 92 to 138 kg P_2O_5 ha^{-1}, the fresh bulb weight did not change significantly. Thus, the heaviest bulbs were produced already at 92 kg P_2O_5 ha^{-1}. The mean bulb weight of onion plants grown at the rate of 92 kg P_2O_5 ha^{-1} exceeded the fresh bulb weight of onion plants grown at the rates of 46 and 0 kg P_2O_5 ha^{-1} by about 11 and 33%, respectively (Table 2). The significant increase in fresh bulb weight in response to the increased phosphorus level might be attributed to the role phosphorus played in improving the carbohydrate content of the plants and extending root growth, which increased the diameter and length of blubs and ultimately blub size (Jawar et al., 2016). This finding is also in accordance with that of Lemma and Shimeles (2003) who reported that at 92 kg P_2O_5 ha^{-1}, there was better vegetative growth and the average bulb weight was 49 g/bulb at Melkassa. Similar results were also reported by Shaheen et al. (2007) who obtained that the highest application of phosphorus (92 kg ha^{-1}) fertilizer had a significant effect on the productivity of onion plant, hence increased average bulb weight.

Total biomass yield: The analysis of variance revealed that the main effect of phosphorus fertilizer application significantly ($P<0.05$) influenced the total biomass yield of onion, compared to the control treatment. On the other hand, neither the main effect of seedling age nor its interaction with phosphorus influenced the total biomass yield of the crop. Increasing the rate of phosphorus from 0 to 46 kg P_2O_5 ha^{-1} significantly increased the total biomass yield (Table 2). However, further application beyond 46 kg P_2O_5 ha^{-1} did not significantly increase the biomass yield. The total biomass yield of onion plants grown at a rate of 46 kg P_2O_5 ha^{-1} exceeded the total biomass yield of onion plants grown at rates of 0 kg P_2O_5 ha^{-1} by about 12%. The highest total biomass yield was obtained from the highest phosphorus rate (138 kg ha^{-1}) though not significantly different from those obtained at 46 and 92 kg P_2O_5 ha^{-1}, whereas the lowest total biomass

Table 2. Main effects of seedling age and phosphorus fertilizer rates on fresh bulb weight, total biomass yield, bulb dry weight and unmarketable and under sized bulb yield of onion grown at Alage.

Treatment	Fresh bulb weight (g)	Total biomass yield (t ha^{-1})	unmarketable bulb yield (t ha^{-1})	Bulb dry matter fraction (%)	Neck thickness (cm)
Seedling age					
6	81.28^{b}	47.32	1.88	10.98^{b}	1.03
7	79.19^{b}	48.69	2.08	11.89^{a}	1.03
8	86.27^{a}	52.64	2.03	12.36^{a}	1.02
LSD(0.05)	3.62	-	-	0.9	-
Significance level	**	NS	NS	*	NS
P_2O_5 (kg ha^{-1})					
0	66.84^{c}	43.69^{b}	2.66^{a}	10.68^{b}	0.97
46	80.42^{b}	49.05^{ab}	2.08^{ab}	10.91^{b}	1.03
92	88.89^{a}	52.61^{a}	1.80^{bc}	12.33^{a}	1.02
138	92.83^{a}	52.86^{a}	1.45^{c}	13.05^{a}	1.08
LSD(0.05)	4.18	7.21	0.57	1.04	-
Significance level	***	*	**	**	NS
CV (%)	6.13	17.51	34.75	10.64	10.55

Means followed by the same letters within a column are not significantly different at ($P < 0.05$).

yield was obtained under the control treatment. This result is in line with that of Shaheen et al. (2007) who reported that application of phosphorus had a major effect on the productivity of onion plant, hence increased total biomass yield. This result is in disagreement with that of Abdissa (2008) who reported that phosphorus has shown non-significant effect on total dry biomass yield of onion; thus, absence of response to phosphorus in the previous study might be due to sufficient amount of available P which was found in the soil of the experimental site.

Unmarketable bulb yield: The analysis of variance indicated that phosphorus application rate had significant ($P<0.01$) effect on unmarketable bulb yield of onion. However, neither the main effect of seedling age nor its interaction with phosphorus affected unmarketable bulb yield. The result of the study indicated that increasing the rates of application of phosphorus decreased the unmarketable bulb yield per hectare. Among all phosphorus rates the highest unmarketable bulb yield was recorded in the unfertilized plots, whereas the lowest was recorded at 138 kg ha^{-1} phosphorus rate though this was statistically at par with 92 kg ha^{-1} (Table 2). High unmarketable yield observed in low rates of phosphorus application have been associated with early bulb formation, severe stunting, and fewer large sized bulbs than those under high phosphorus rates. This result is contrary with that of Tibebu et al. (2014) who stated that phosphorus had no significant effect on unmarketable yield of onion that might be due to adequate amount of available P which was found in the soil of the experimental site.

Total bulb yield: The main effect of seedling age as well as that of phosphorus significantly ($P < 0.001$) influenced the total bulb yield of onion. Additionally, the interaction effect of seedling age and phosphorus application rate significantly ($P < 0.05$) influenced the total bulb yield of the onion. At 6 weeks of seedling age, total bulb yield was significantly improved at 92 kg ha^{-1} P_2O_5 rate. Further increasing phosphorus up to 138 kg ha^{-1} showed a decline in the total bulb yield by 29%, however, no significant difference was showed among the two phosphorus rates except those unfertilized plots and 92 kg P_2O_5 ha^{-1}, which were at par. Under 7 weeks of seedling age, the highest and lowest total bulb yields were recorded at 92 kg ha^{-1} phosphorus and control treatments, respectively. However, no significant difference was recorded among 46 and 138 kg P_2O_5 ha^{-1} treatments. In the 8 weeks of seedling age, P application rate at 92 and 138 kg ha^{-1} improved total bulb yield by about 36 and 42%, respectively compared to that of untreated treatment. However, the two phosphorus rate (92 and 138 kg ha^{-1}) had no significant difference at the same seedling age of 8 weeks (Table 3). Generally, increased application rate of phosphorus produced higher total bulb yields with optimum seedling age (8 weeks seedling age). The high total bulb yield produced due to phosphorus application and old seedling age might be because of increase in photosynthetic area of the plant (plant height and number of leaves) which in turn increased the amount of assimilate that could be partitioned to the storage organs (increased bulb

Table 1. Interaction effects of seedling age and phosphorus rates on total bulb yield (t ha^{-1}) of onion grown at Alage.

Seedling age (weeks)	Total bulb yield (t ha^{-1})				Harvest Index (%)			
	P_2O_5(kg ha^{-1})				P_2O_5(kg ha^{-1})			
	0	46	92	138	0	46	92	138
6	24.27^{f}	35.48^{de}	45.24^{ab}	35.01^{de}	77.55^{c}	87.67^{ab}	90.07^{a}	89.7^{a}
7	31.83^{e}	39.11^{cd}	43.43^{bc}	39.52^{cd}	83.83^{b}	91.03^{a}	90.1^{a}	91^{a}
8	35.47^{de}	39.54^{cd}	48.49^{a}	50.6^{a}	89^{a}	90.2^{a}	89.15^{a}	91.75^{a}
LSD(0.05)	4.43				3.00			
Significance level	*				*			
CV (%)	9.66				3.5			

Means followed by the same letters within a column or row are not significantly different at (P<0.05).

diameter and average bulb weight). This may lead to improved carbohydrate content of the plants and extended root growth, which consequently increased the total bulb yield. In harmony to this result, Verma et al. (1971) reported that transplanted onion at 8 weeks old gave the highest yield of bulb.

Harvest index: The analysis of variance showed that means of harvest index was significantly ($P < 0.05$) affected by the interaction effect of seedling age and phosphorus rate. Moreover, harvest index was significantly influenced by the main effects of seedling age ($P < 0.01$) and phosphorus application ($P < 0.001$). Generally, increased rate of phosphorus produced higher harvest index with increased age of seedling (Table 3). The highest harvest index was recorded under the 8 weeks of seedling age combined with application of 138 kg P_2O_5 ha^{-1}, while the lowest value was obtained from transplanting of 6 weeks of seedling age with the control treatment. At 6 week seedling age, harvest index was significantly improved at 92 P_2O_5kg ha^{-1} rate, further increasing of phosphorus up to 138 kg ha^{-1} at this seedling age showed a decline in the harvest index. However, statically no significant difference was observed among the two phosphorus rate except those untreated plots and 46 kg ha^{-1} which were at par. Under 7 week seedling age, the highest and lowest harvest indices were recorded at 46 kg ha^{-1} phosphorus and control plots, respectively. However, no significant difference was recorded among 46, 92 and 138 kg P_2O_5 ha^{-1} P rates. In the 8 weeks of seedling age, no significant difference was recorded among 0, 46, 92 and 138 kg P_2O_5 ha^{-1} treatments. Over all, differences among the three seedling age levels become progressively narrower with increasing rates of P application. The high harvest index produced due to P application and higher seedling age might be because of increased photosynthetic area of the plant (height of plants and number of leaves) which increased the amount of assimilate that could be partitioned to the storage organs (increased bulb length and average bulb weight) which consequently increased the harvest index.

Quality parameters

Bulb dry matter fraction

The main effect of seedling age ($P < 0.05$) and phosphorus ($P < 0.001$) significantly affected mean bulb dry matter fraction of onion plants, however, the interaction effect did not show significant differences ($P > 0.05$). This study indicated that, significant differences were observed among the seedling age levels, in such a way that the 8 weeks of seedling age gave the highest onion bulb dry matter fraction, while the lowest bulb dry matter fraction was obtained under 6 weeks of seedling age. However, no statically significant difference was observed between 7 and 8 weeks of seedling age (Table 2). The dry matter fraction for different age of seedlings varied possibly due to variation of growth patterns and photosynthesis at growing phases. The results of the present study are in agreement with Latif et al. (2010), Sultana (2015) and Bhonde et al. (2001) who reported that dry matter content of onion bulb was significantly influenced by the age of seedling. This might be due to the fact that the optimum age of seedlings planted had better growth, which resulted in higher production of dry matter content of bulb (Sultana, 2015). This result is also consistent with the findings of Muhammad et al. (2016) who reported that the seedling transplanted at 60 days have high dry matter percentage as compared to the seedling transplant in early stage and it might be attributed to the fact that as the bulb size decreased quantity of water content also decreased resulting in high percentage of dry matter. Increasing the rate of phosphorus from nil to 46 kg P_2O_5 ha^{-1} not significantly increased the bulb dry matter fraction of onion. But, increasing the rate of phosphorus further from 46 to 92 kg ha^{-1} increased the dry matter fraction of onion plants. And also the mean dry matter fraction of plants did not show significant difference as further increase in phosphorus

Table 4. Interaction effects of seedling age and phosphorus rates on marketable bulb yield (t ha^{-1}) of onion grown at Alage.

Seedling age (weeks)	P_2O_5(kg ha^{-1})			
	0	46	92	138
6	21.63^{g}	33.25^{def}	43.22^{ab}	33.17^{def}
7	28.09^{f}	36.07^{cde}	40.79^{bc}	37.51^{cd}
8	32.08^{ef}	36.78^{cde}	45.84^{a}	48.33^{a}
LSD(0.05)		4.43		
Significance level		*		
CV (%)		10.26		

Means followed by the same letters within a column or row are not significantly different at (P<0.05).

rate from 92 to 138 kg ha^{-1}. Thus, the mean dry matter fraction of onion treated with phosphorus at the rate of 92 kg ha^{-1} exceeded the bulb dry matter of onion plants treated with nil and 46 kg P_2O_5 ha^{-1} by about 15 and 13%, respectively. Similar observations were reported by Woldetsadik (2003) who stated that on a clay soil in a sub-humid tropical environment of Ethiopia in shallot (*Allium ascalonicum*) crop, increased application of P slightly increased bulb dry matter content of onion. Similarly, Tibebu et al. (2014) reported that the rate of P application increased dry matter content of onion.

Neck thickness

Seedling age and phosphorus fertilization and their interaction did not significantly ($p > 0.05$) affect the formation of neck thickness of onion (Table 2). This could be due to the minimal direct effect of fertilization in the formation of thick-necked bulbs. Brewester (1987) reported that neck-thickness is a physiological event that is influenced by seasons, sites and cultivars, not by fertility.

Marketable bulb yield

Marketable bulb yield of onion was significantly affected ($P < 0.001$) by the seedling age and phosphorus rate. Similarly, significant interaction effect of seedling age and phosphorus was observed on the marketable bulb yield of onion ($P<0.05$).

Under 6 weeks of seedling age, marketable bulb yield increased by about 100% at 92 kg ha^{-1} phosphorus compared to lowest yield recorded from untreated plots (Table 4). Further increase of phosphorus to 138 kg ha^{-1} did not significantly show variation, rather it showed a drop by about 30% and leveled off with yields from control plots and those fertilized at 46 kg ha^{-1} phosphorus. At 7 weeks of seedling age, the highest marketable bulb yield was produced at 92 kg ha^{-1} P rate while the lowest marketable bulb yield was obtained from control treatments. Significant differences were recorded among yields at 46, 92 and 138 kg ha^{-1} P rate under the 7 weeks age. At 8 weeks of seedling age, the control plot had notably reduced yield of marketable bulb as compared to the three phosphorus rates. The highest marketable bulb yield was recorded at 8 weeks of seedling age combined with 138 kg ha^{-1} phosphorus rate; though statically at par to that obtained from 92 kg P_2O_5 ha^{-1} under similar seedling age (Table 4).

From the present result it can be deduced that old seedling age and higher phosphorus rate help to increase the vegetative growth of the plant which has improved assimilate availability for storage and led to an increased average bulb weight that gave an advantage to increase the marketable bulb yield.

Partial budget analysis

The partial budget analysis revealed that the highest net benefit of Birr 152829 was recorded from the combination of 138 kg P_2O_5 ha^{-1} and 8 weeks of seedling age with marginal rate of 1481%. This was followed by net benefit of Birr 145372.3 from the phosphorus rate of 92 kg P_2O_5 ha^{-1} and 8 weeks of seedling age with the marginal rate of return of 5657%. This means that for every Birr 1.00 invested in 92 kg P_2O_5 ha^{-1} and 8 weeks of seedling age, producers can expect to recover the Birr 1.00 and obtain an additional 56.57 Birr. Whereas, the lowest net benefit (Birr 69200 ha^{-1}) was recorded from control treatments (0 kg P_2O_5 ha^{-1}) combined with 6 weeks of seedling age (Table 5).

The minimum acceptable marginal rate of return (MRR %) should be between 50 and 100% (CIMMYT, 1988). Thus, the current study indicated that marginal rate of return is higher than 100% (Table 6). Hence, the most economically attractive yield of the onion crop in the study area was that the combinations of 92 kg P_2O_5 ha^{-1} application and 8 weeks of seedling age with low cost of production and higher benefits. The yield from the combination of 138 kg P_2O_5 ha^{-1} with 8 week old seedlings still meets the 100% marginal rate of return

Table 5. Partial budget analysis for phosphorus rate and seedling age experiments of onion at Alage.

Treatments		Average yield (t ha^{-1})	Adjusted yield (t ha^{-1})	Gross benefit (Birr ha^{-1})	Total cost that vary (Birr ha^{-1})	Net benefit (Birr ha^{-1})
Seedling age (weeks)	P rate (kg ha^{-1})					
6	46	33.25	26.6	106400	803.7	105596.3^{D}
7	46	36.07	28.86	115440	803.7	114636.3^{D}
8	46	36.78	29.42	117680	803.7	116876.3
6	92	43.22	34.58	138320	1307.4	137012.3^{D}
7	92	40.79	32.63	130520	1307.4	129212.3^{D}
8	92	45.84	36.67	146680	1307.4	145372.6
6	138	33.17	26.54	106160	1811	104349^{D}
7	138	37.51	30	120000	1811	118189^{D}
8	138	48.33	38.66	154640	1811	152829
6	0	21.63	17.3	69200	-	69200^{D}
7	0	28.09	22.47	89880	-	89880^{D}
8	0	32.08	25.66	102640	-	102640^{D}

Table 6. Marginal analysis, seedling age and phosphorus rates experiment in Alage.

Treatments		Total cost that vary (Birr ha^{-1})	Marginal cost (Birr ha^{-1})	Net benefit (Birr ha^{-1})	Marginal net benefit (Birr ha^{-1})	Marginal rate of return (%)
Seedling age	P rate (kg ha^{-1})					
8	46	803.7	-	116876.3	-	-
8	92	1307.4	503.7	145372.6	28496	5657
8	138	1811	503.6	152829	7456.4	1481

threshold value. However, this treatment will not be a viable option because the yield from this combination was statistically at par with that obtained from 92 kg P_2O_5 ha^{-1} and 8 weeks old age.

Conclusions

The analysis of variance showed that plant height, leaf length, days to maturity, bulb length, fresh bulb weight, and bulb dry matter fraction were significantly influenced by the main effect of seedling age and phosphorus fertilizer rates. However, leaf number per plant, bulb diameter, total biomass yield and unmarketable bulb yield were significantly affected only by the main effects of different rates of phosphorus fertilizer. Seedling age and P fertilizer rate as well as their interaction did not significantly affect the formation of neck thickness of onion. From this study, significantly taller plant height, leaf number per plant, leaf length and early bulb maturity was obtained at the seedling age of 8 weeks and 92 kg ha^{-1}phosphorus rate. However, bulb length, fresh bulb weight, total biomass yield and bulb dry matter fraction were recorded in the treatments of 8 weeks of seedling age and 138 kg ha^{-1} phosphorus rate. However, the result of this study showed that at 92 and 138 kg P_2O_5 ha^{-1} rate there was no significant variation in each parameter.

Therefore, the study showed that, the highest yield of marketable and total bulb yield of Bombay Red onion variety were produced at treatment combination of 8 weeks of seedling age with 138 kg ha^{-1} P rate, but no significant difference was observed in these parameters at 92 kg P_2O_5 ha^{-1} combinations with same seedling age. However, the combination of 8 weeks of seedling age fertilized with 92 kg ha^{-1} P rate also gave statistically comparable yield to the highest value. Therefore, from the present study it can be concluded that, the most economically attractive yield of the onion crop in the study area was obtained by the combinations of 92 kg P_2O_5 ha^{-1} applications and 8 weeks of seedling age with low cost of production and higher benefits.

Since this experiment is a one-year study in a single environment, further research over locations and years is warranted to confirm the present results.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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Genotype and genotype by environment interaction and grain yield stability of medium maturity groups of soybean [*Glycine max* (L.) Merrill] varieties in Western Oromia, Ethiopia

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The objectives of this study were to identify high yielding and stable medium maturity soybean varieties across environment and examine the influence of genotype × environment interaction (GEI) on grain yield of soybean varieties in western Oromia. Seven early soybean varieties were evaluated at five locations (Bako, Gute, Billo, Chewaka and Uke) using randomized complete block design (RCBD) with three replications for two consecutive years (2016 and 2017). Combined analysis of variance showed that grain yield was significantly (P<0.01) affected by environments, genotypes and GEI. The environment, genotype and genotype by environment interaction accounted for 57.4, 20.9 and 19.8% variations, respectively. The first two principal components ($IPCA_1$ and $IPCA_2$) were used to create a two-dimensional genotype and genotype by environment interaction (GGE) biplot and explained 68.9 and 15.6% of the total sums of squares of GEI, respectively. According to the average environment coordination (AEC) views of the GGE-biplot, soybean variety Didhessa and Hawassa-04 were identified as the most stable and high yielding varieties. In addition, Didhessa and Hawassa-04 also showed better stability performance according to AMMI stability value (ASV), genotypic selection index (GSI), Wricke's ecovalence and cultivar superiority measure among the evaluated varieties whereas variety Davis and AFGAT were identified as the least stable and low yielding variety. Therefore, among medium maturing soybean varieties, Didhessa, Hawassa-04 and Cheri were recommended for further production in most soybean growing areas of western Oromia.

Key words: Additive main effects and multiplicative interaction (AMMI), AMMI stability value (ASV), cultivar superiority measure, genotype, genotypic selection index (GSI).

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is a legume native to East Asia perhaps in North and Central China (Laswai et al., 2005) and it is grown for edible bean, oil and protein around the world. Soybean is found in family Fabaceae

and species *G. max* (Shurtleff and Aoyagi, 2007). Soybean is one of the most important oil grain legume crops in the world. In the International trade market, soybean ranks number one among the major oil crops with an average protein content of 40% on dry matter basis. It has the highest protein content of all field crops and is the second only to groundnut in terms of oil content (20%) among the food legumes. Dugje et al. (2009) reported that soybean is more protein rich than any of the common vegetable or legume food sources in Africa. Soybean is a promising pulse crop proposed for alleviation of acute shortage of protein and oil worldwide (Mahamood et al., 2009). It is used as a good source of unsaturated fatty acids, minerals (Ca and P) and vitamins A, B, C and D (Alam et al., 2009). Zerihun et al. (2015) indicated that soybean in Ethiopia could be grown between 1300 and 1800 m altitude with annual rain fall of 900 to 1300 mm, an average annual temperature between 20 and 25°C and soil pH of 5.5.

Soybean is classified in different groups such as early, medium and late maturing varieties. A variety is classified to a specific maturity groups according to the length of period from planting to maturity. This phenological attribute is determined by two abiotic factors: photoperiod and temperature (Mourtzinis and Conley, 2017), and these factors can dictate the most suitable maturity groups of soybean varieties for a particular geographical location. Therefore, identification of different maturity groups of soybean varieties that fit specific agro-ecologies of western Oromia is an alternative option to boost soybean productivities.

In Ethiopia, soybean is a multipurpose crop, which can be used for a variety of purposes including preparation of different kinds of soybean foods, animal feed, soy milk, raw material for the processing factories like tasty soya, fafa food factories, etc. Currently, there are also factories producing oil from soybean showing increasing importance of soybean in the country. It also counter effects depletion of plant nutrients especially nitrogen in the soil resulting from continuous mono-cropping of cereals, especially maize and sorghum, thereby contributing to increasing soil fertility (Mekonnen and Kaleb, 2014). Its area of production is increasing and according to CSA (2016) report, soybean was produced on about 38,166.04 ha of land and 81241.833 tons produced in 2015/16 main cropping season with the productivity of 2.1 t ha^{-1}; which is low as compared to world average of 2.6 t ha^{-1}. This low yield may be attributed to a combination of several production constraints among which low soil fertility, lack of high yielding varieties, periodic moisture stress, diseases and insect-pests, weeds and poor crop management practices play a major role (Georgis et al., 1990).

Genotypes exhibit fluctuating yields when grown in different environments or agro-climatic zones. This complication demonstrates the superiority of a particular genotype. Multi-environment yield trials are crucial to identify adaptable high yielding cultivars and discover sites that best represent the target environment (Dabessa et al., 2016). It was also reported by Yazici and Bilir (2017). Poor response of genotypes to different environmental condition is the result of genotype and genotype by environment interaction (GGE). The information and understanding of GGE is good to have varieties that gives permanently high yield in wider range of environments and to increase efficiency of breeding program and selection of best genotypes. Knowledge and information of GGE permit for judging the performance of genotypes in evaluated environments. The level of yield variation of genotypes across environments resulted from genotype, environment and genotype by environment interaction (Amare and Tamado, 2014; Funga et al., 2017). Thus, multi-environment trials (MET) are required to identify genotypes that have the specific and the general adaptability in tested environments. In western Oromia, the yield of medium soybean variety is very low due to different biotic and abiotic factors. Therefore, the objectives of this study was to identify high yielding and stable medium maturity soybean varieties across environment and consider the effect of genotype × environment interaction (GEI) on grain yield of soybean varieties.

MATERIALS AND METHODS

Seven medium maturity groups of released soybean varieties (Clark 63k, Davis, Cheri, AFGAT, Didhessa, Hawassa-04 and Wello) were evaluated at six locations for two consecutive years during 2016 and 2017 main cropping season (Table 1). The study sites included Billo and Gute during 2016, Chewaka and Uke during 2017 main season and Bako during 2016 and 2017 (Table 2). The experimental land was ploughed, disked and harrowed by tractor. The first ploughing was done before on-set of rainfall. The plantings were done in mid-June at each location using a randomized complete block design with three replications. Each plot consisted of four rows of 4 m length with 40 and 10 cm spacing between rows and seeds, respectively. The two middle rows were used for data collection and harvested at maturity. Fertilizer was applied at the rate of 100 kg NPS ha^{-1} during planting time. All other management practices were applied as per the recommendations.

Multivariate method, Additive Main Effects and Multiplicative Interaction (AMMI) model was used to assess GEI pattern. AMMI model is expressed as:

$$Y_{ger}=\mu+ag+ße+\sum n\lambda n\gamma gnden+eger+\rho ge \quad (1)$$

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Table 1. Pedigree, origin, area of adaptation and year of release of soybean varieties used for the study.

Variety	Pedigree	Source of materials	Year of release	Adaptation altitude (masl)	RF (mm)	Maturity date
Clark 63k	NI	HwARC/SARI	1981	1000-1700	520-1500	110-120
Davis	NI	HwARC/SARI	1981	1000-1700	400-700	115-125
Cheri	IBP-81EP7	BARC/OARI	2003	1300-1850	900-1300	110-120
Afgat	TGX-1892-10F	HwARC/SARI	2007	520-1800	750-1300	110-120
Didessa	PR-149-81-EP-7-2	BARC/OARI	2008	1200-1900	1000-1200	115-125
Hawassa-04	AGS-7-1	HwARC	2012	1200-1700	500-1300	110-120
Wello	TGX-1895-33F	SARI/ARARI	2012	520-1800	520-1200	115-125

NI: Not indicated.

Table 2. Environments used in the study and their main characteristics.

Location	Year	Longitude	Latitude	Altitude (masl)	RF (mm)	Soil type
Bako	2016 & 2017	37°09'E	09°06'N	1650	1431	Sandy-clay
Gute	2016	E:036°38.196’	N:09°01.061’	1915	NI	Clay
Billo	2016	E:037°00.165’	N:09°54.097’	1645	1500	Reddish brown
Chewaka	2017	036.11703E	09.98285N	1259	NI	Clay loam
Uke	2017	E:036°32..391’	N:09°25.082’	1319	NI	Sandy loam

NI: Not indicated.

where Yger is the observed yield of genotype (g) in environment (e) for replication (r); additive parameters: μ is the grand mean, ag is the deviation of genotype g from the grand mean, and ße is the deviation environment e; multiplicative parameters: λn is the singular value for IPCA, γgn is the genotype eigenvector for axis n, den is environment eigenvector; eger is error term and ρge is PCA residual.

Accordingly, genotypes with low magnitude regardless of the sign of interaction principal component analysis scores have general or wider adaptability while genotypes with high magnitude of IPCA scores have specific adaptability (Gauch, 1992; Umma et al., 2014).

AMMI stability value of the i^{th} genotype (ASV) was calculated for each genotype and each environment according to the relative contribution of $IPCA_1$ to $IPCA_2$ to the interaction SS as follows (Purchase et al., 2000):

$$ASV_i = \sqrt{\left[\frac{SS_{IPCA1}}{SS_{IPCA2}}[IPCA1_{score}]\right]^2 + [IPCA2_{score}]^2} \quad (2)$$

where SS_{IPCA1}/SS_{IPCA2} is the weight given to the $IPCA_1$ value by dividing the $IPCA_1$ sum of squares by the $IPCA_2$ sum of squares.

Based on the rank of mean grain yield of genotypes (RY) across environments and rank of AMMI stability value (RASV) a selection index called genotype selection index (GSI) was calculated for each genotype, which incorporates both mean grain yield (RY) and stability index in single criteria (GSI) as (Purchase et al., 2000):

$$GSI = RASV + RY \quad (3)$$

Wricke’s ecovalence (Wi)

Wricke (1962) proposed using the contribution of each genotype to the G×E interaction sum of squares as a stability parameter.

$$Wi = \frac{P}{(P-2)(q-1}\sum_{j=1}^{q}(Xij - \bar{X}.\mathrm{i} - \bar{\mathrm{X}}.\mathrm{j} + \bar{\mathrm{X}}..)2 \quad (4)$$

where x_{ij} is the mean performance of genotype i in the j^{th} environment, xi. and x.j are the marginal mean of genotype i and environment j, respectively and x.. is the overall mean. Thus, genotype with a low W_i value are stable.

Lin and Binns cultivar superiority measure

A cultivar-superiority measure was used to calculate stability coefficients for genotype by environment data of each genotype. It is computed as the sum of the squares of the differences between its mean in each environment and the mean of the best genotype there, divided by twice the number of environments (Lin and Binns, 1988).

GGE was used to make judgment about the performance of soybean genotypes in different environments. The environmental effects were removed from the data and results obtained from the data were used to calculate environment and variety scores and these scores were used to plot the standard principal component bi-plots (Yan and Kang, 2003). Analysis of variance (ANOVA) and AMMI analysis and GGE bi-plots were performed using Gen Stat 18th edition statistical package (GenStat, 2016).

Table 3. Combined analysis of variance for grain yield of medium soybean varieties evaluated at six environments in western Oromia.

Source of variation	Degree freedom	Mean square
Environments	5	6389445**
Genotypes	6	1947323**
Block within environment	2	6355^{ns}
Interaction	30	367131**
Error	82	12501
LSD (0.05)	181.6	-
CV (%)	5	-

LSD: Least significant differences, CV: coefficient of variation, **Significant at P = 0.01, ns: non-significant.

RESULTS AND DISCUSSION

Combined ANOVA

There were statistically significant differences (P<0.01) among evaluated soybean varieties, environments and their interaction for seed yield (Table 3). This suggests the existence of genetic variation among the soybean varieties and possibility to select high yielding and stable variety (s), the environments are variable and the differential response of soybean varieties across the testing environments. Dabessa et al. (2016) also reported statistically significant difference among groundnut genotypes, respectively.

Performance of soybean varieties across environments

Figure 1A and B shows the performance of soybean varieties at each environments and average mean seed yield of soybean varieties evaluated across six environments in western Oromia, respectively. The pooled mean grain yield ranged from 1683 to 2720 kg ha^{-1} (Figure 1B). Among all varieties, Davis was the lowest yielder. The highest grain yield was obtained from Hawassa-04 variety (2720 kg ha^{-1}) followed by Didessa (2436 kg ha^{-1}). This differential yield response of soybean varieties could be due to their genetic potential. Hawassa-04 was the top ranking genotype at Bako (2016 and 2017) and Gute, while Clark 63k, Cheri and Didessa gave the highest yield at Billo, Chewaka and Uke, respectively (Figure 1B). The difference in yield response of medium soybean varieties across the test environments were the results of changing genotypes from one area to the other areas, that is, showed high crossover type of genotype by environment interaction. In line with this result, Tolessa and Gela (2014) reported variable yield response of common bean genotypes evaluated across different locations in Ethiopia.

AMMI model analysis

The AMMI model ANOVA for grain yield is shown in Table 4. This analysis also revealed the presence of highly significant (P< 0.01) differences among medium soybean varieties for grain yield performance. From the total treatment, sum of squares, the largest portion was due to environments main effect (57.4%) followed by varieties main effect (20.9%) and the effect of genotype by environment interaction was 19.8%. This suggests the existence of a large amount of inconsistent response among the evaluated soybean varieties to changes in growing environments. Similar result was reported by Dabessa et al. (2016). Considerable percentage of GEI was explained by $IPCA_1$ (8.6%) followed by $IPCA_2$ (6.3%) and therefore used to plot a two dimensional GGE biplot. Amare and Tamado (2014) indicated the most accurate model for AMMI can be forecasted by using the first two IPCA.

In the first four AMMI selection of genotypes, Hawassa-04 took the first position in Bako, Gute and Billo while Didhessa took the second best position in Uke, Bako, Gute and Billo environments (Table 5). Accordingly, Hawassa-04 and Didhessa varieties revealed static stability as compared to other varieties, which is a desirable characteristic for crop production. The relative static performance of Hawassa-04 and Didhessa varieties in different environment is an indication of general adaptability of these varieties. AFGAT and Wello varieties took the first position at Chewaka and Uke showing uniform yield performance in the particular environment (Table 5). The report indicated that the interaction pattern of some locations across crop species is consistent so that they are highly predictable in year to year interaction with genotypes (Ebdon and Gauch, 2002).

AMMI biplot analysis

AMMI biplot graph (Figure 2) with X-axis plotting $IPCA_1$

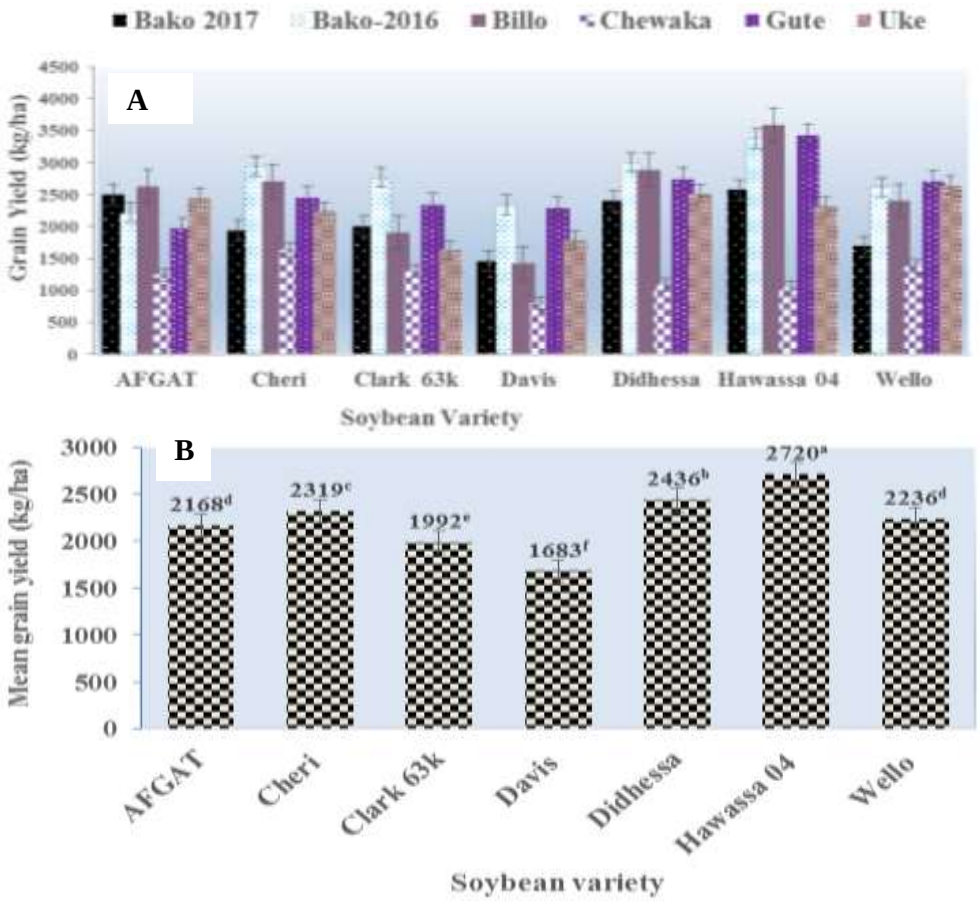

Figure 1. Performance of (A) medium maturity groups of soybean varieties at each environments, (B) mean performance of soybean varieties across environments. Bars followed by same letters are not significantly different from each other at LSD (0.05).

Table 4. Partitioning of the explained sum of square (SS) and mean square (MS) from AMMI analysis for grain yield of seven soybean varieties.

Source of variation	DF	Sum of square	Explained SS (%)	Mean square
Total	125	55682927	-	445463
Treatments	41	54645104	-	1332807**
Genotypes	6	11683938	20.9	1947323**
Environments	5	31947226	57.4	6389445**
Block	12	251654	0.45	20971
Interactions	30	11013940	19.8	367131**
IPCA 1	10	4760206	8.6	476021**
IPCA 2	8	3486895	6.3	435862**
Residuals	12	2766840	-	230570
Error	72	786169	-	10919

ns: Non-significant, **significant at 1% and *significant at 5% probability level. SS: Sum of square, DF: degree of freedom.

and Y-axis plotting $IPCA_2$ scores illustrate stability, adaptability and high yielding of soybean varieties to the testing environments. It has been reported that the $IPCA_1$ scores of a genotypes in AMMI analysis are an indication

Table 5. First four AMMI selections per environment.

Environment	Mean yield (kg ha^{-1}	Genotype rank			
		1	2	3	4
Chewaka	1209	Wello	Cheri	AFGAT	Didhessa
Uke	2224	AFGAT	Didhessa	Cheri	Hawassa-04
Bako-2016	2754	Hawassa-04	Didhessa	Cheri	Wello
Bako 2017	2081	Hawassa-04	AFGAT	Didhessa	Cheri
Gute	2559	Hawassa-04	Didhessa	Cheri	Wello
Billo	2505	Hawassa-04	Didhessa	AFGAT	Cheri

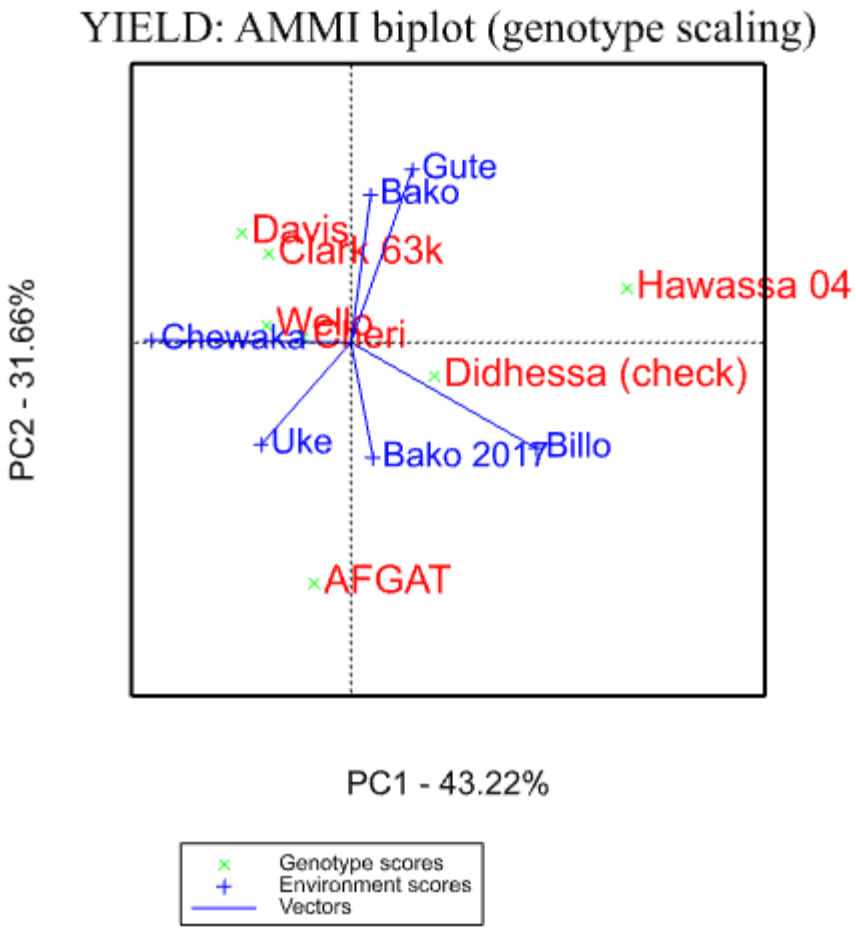

Figure 2. AMMI biplot showing "which won where" and stable soybean varieties evaluated at six environments in western Oromia.

of the stability or adaptation over environments (Alberts, 2004). It is further stated that the greater the IPCA scores, negative or positive, the more specific adapted is a genotype to certain environments. The more the IPCA scores approximate to zero, the more stable or adapted the genotypes is over all the environments sampled. According to AMMI biplot, Environments Bako and Gute relatively showed high IPCA scores and contributed largely to GEI. Bako and Gute environments were conducive for best performing soybean varieties. Environments Chewaka and Uke are the low yielding environment for most of the varieties (Figure 2). Varieties

Table 6. AMMI stability value, genotype selection index and ranks based on grain yield of seven medium soybean varieties evaluated at six locations during 2016 and 2017 seasons.

Variety	Yield	ASV	RY	RASV	GSI
AFGAT	2167.6	29.08	5	5	10
Cheri	2318.9	9.20	3	2	5
Clark-63k	1991.9	24.46	6	4	10
Davis	1683.4	3.31	7	1	8
Didhessa (check)	2436.3	40.62	2	6	8
Hawassa-04	2719.6	21.39	1	3	4
Wello	2236.3	42.62	4	7	11

ASV: AMMI stability value, RY: rank of yield, RASV: rank of AMMI stability value, GSI: genotype selection index.

Table 7. Stability analysis of Cultivar superiority index, static stability and wrikles ecovalence values of medium soybean varieties evaluated in western Oromia.

Variety	Cultivar superiority	Rank	Wricke's Eco valence	Rank
AFGAT	382694	5	864972	6
Cheri	207273	3	230634	2
Clark-63k	489342	6	466509	5
Davis	813077	7	420541	4
Didhessa	121320	2	158038	1
Hawassa-04	38822	1	1120645	7
Wello	282916	4	409974	3

Davis, Clark 63k and Wello were intended to low yielding environment (Figure 2). Based on the IPCA score, AFGAT and Davis were not stable varieties and as well performed under low yielding environments. Dhidhessa variety revealed more static performance across environments in comparison to other soybean varieties. Varieties Wello and Cheri were adapted to low yielding environments and also relatively stable (Figure 2). Dhidhessa and Hawaassa-04 varieties have relatively lower IPCA by which they proved to have best grain yield stability than other varieties (Figure 2). Hawassa-04 variety had the highest grain yield followed by Dhidhessa variety. Similar results were also reported by Temesgen et al. (2014) on linseed and Niger seed in Western Ethiopia.

AMMI stability value and genotype selection index

Analysis of AMMI stability value (ASV) and genotype selection index (GSI) with their ranking for seven soybean varieties are shown in Table 6. According to ASV result, genotype with least ASV value is the most stable (Purchase et al., 2000). Accordingly, Hawassa-04, Didhessa and Cheri were the most stable, but Clark-63k and Davis showed dynamic stability. This method is vital to measure and rank varieties based on seed yield stability. The summation of rank of ASV and rank of yield are used to calculate GSI. The genotype with least GSI is considered as the most stable with high grain yield (Dabessa et al., 2016). According to GSI, the best variety for choice of high seed yield and general adaptation was Hawassa-04, Cheri and Didhessa, respectively.

Stability analysis using Wricke's ecovalence (wi) and cultivar superiority measure

Stability in performance of soybean varieties across environments using Wricke's ecovalence (Wi) was performed for grain yield. The result showed that Dhidhessa and Cheri were comparatively stable as their contribution to the G×E interaction sum of squares was least (Table 7). On the other hand, AFGAT and Hawassa-04 were unstable in grain yield performance because these genotypes had relatively the highest Wricke's ecovalence (Wi). In line with this result, Gurmu et al. (2009) reported a significant Wricke's ecovalence of twenty soybean genotypes in Southern Ethiopia. According to Lin and Binns (1988) for cultivar superiority measure analysis, the genotype with low or small cultivar superiority measure value is considered to be more

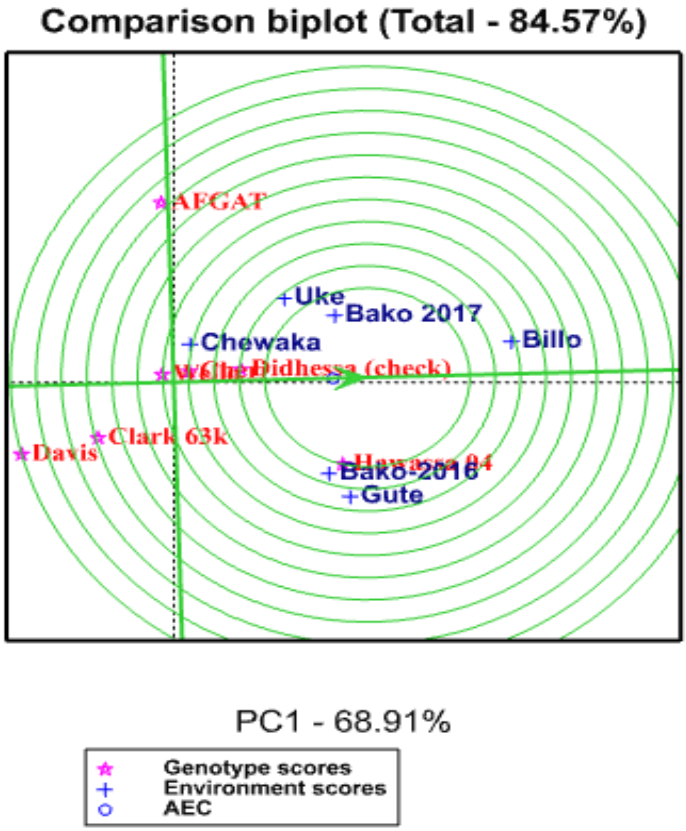

Figure 3. GGE bi-plot based on genotype-focused scaling for comparison of medium soybean varieties for their seed yield potential and stability.

stable. Among studied medium soybean varieties, Hawassa-04 and Didhessa had the smallest cultivar superiority measure values, which showed their best yield performance and seed yield stability (Table 7).

GGE biplot analysis

In GGE biplot (Figure 3), $IPCA_1$ and $IPCA_2$ explained 68.9 and 15.6%, respectively, of soybean varieties by environment interaction and made a total of 84.57%. The other studies conducted on groundnut by Amare and Tamado (2014) and white lupines by Atnaf et al. (2017) explained an interaction of 81.8 and 63.4%, respectively, extracted from $IPCA_1$ and $IPCA_2$. An ideal genotype is defined as genotype which have the greatest IPCA1 score (mean performance) and with zero GEI, as represented by an arrow pointing to it (Figure 3). A genotype is more desirable if it is located closer to the ideal genotype. Thus, using the ideal genotype as the center, concentric circles were drawn to help visualize the distance between each genotype and the ideal genotype. Therefore, the ranking based on the genotype-focused scaling assumes that stability and mean yield are equally important. In this study, Didhessa and Hawassa-04 varieties which fell closest to the ideal genotype was identified as the most desirable varieties as compared to the rest of the tested soybean varieties (Figure 3). Similarly, Dabessa et al. (2016) identified ideal genotype based on the genotype-focused scaling assumes that stability and high mean yield of studied genotypes.

Ideal test environment is an environment which has more power to discriminate genotypes in terms of the genotypic main effect as well as able to represent the overall environments. But such type of environment may not exist in real conditions. Therefore, by assuming a small circle which is located in the center of concentric circles and an arrow pointing on it as ideal environment (Figure 4), it is possible to identify desirable environments which are found closer to the ideal environment (Yan and Rajcan, 2002). Hence, among the testing environments, Billo, which fell near to this ideal environment were identified as the best desirable testing environments in terms of being the most representative of the overall

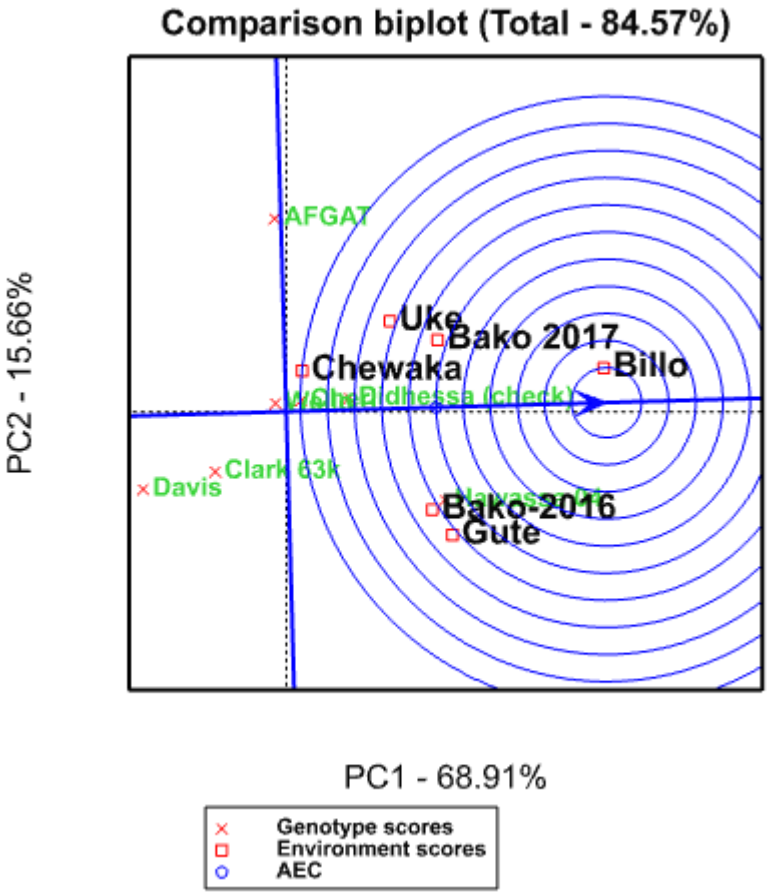

Figure 4. GGE-biplot based on environment-focused scaling for comparison the environments with the ideal environment. PC stands for principal component.

environments and powerful to discriminate soybean varieties.

Discriminating ability and representativeness of environments

Both discriminating ability and representativeness view of the GGE biplot are the most important measures of testing environment, which provide not only valuable but also unbiased information about the tested genotypes (Yan and Kang, 2003). Yan and Tinker (2006) also reported that the length of environmental vector is directly proportional to the standard deviation within the respective environments and help to know the discriminating ability of this target environment, that is, an environment with long environmental vector has high discriminating ability and vice versa. Thus, as shown in Figure 5, the test location (Billo and Gute) were identified as the most discriminating environment as compared to Bako and Uke that were identified as the least discriminating testing environments. Among the testing environments, Chewaka was identified as the least discriminating environment.

Conclusion

Despite its potential and market demand, production of soybean is not yet popularized among farmers in Western Ethiopia. These could be attributed to the lack of information on the effect of genotype, predictable and unpredictable environmental variations and their interaction on yield. Thus, seven medium soybean varieties were tested at six locations under rain fed conditions in western Oromia to determine the effect of genotype, environment, and their interaction and to identify stable ones in yield performance. The environment contributed most to the variability in grain yield. Genotypes Didhessa and Hawassa-04 were close

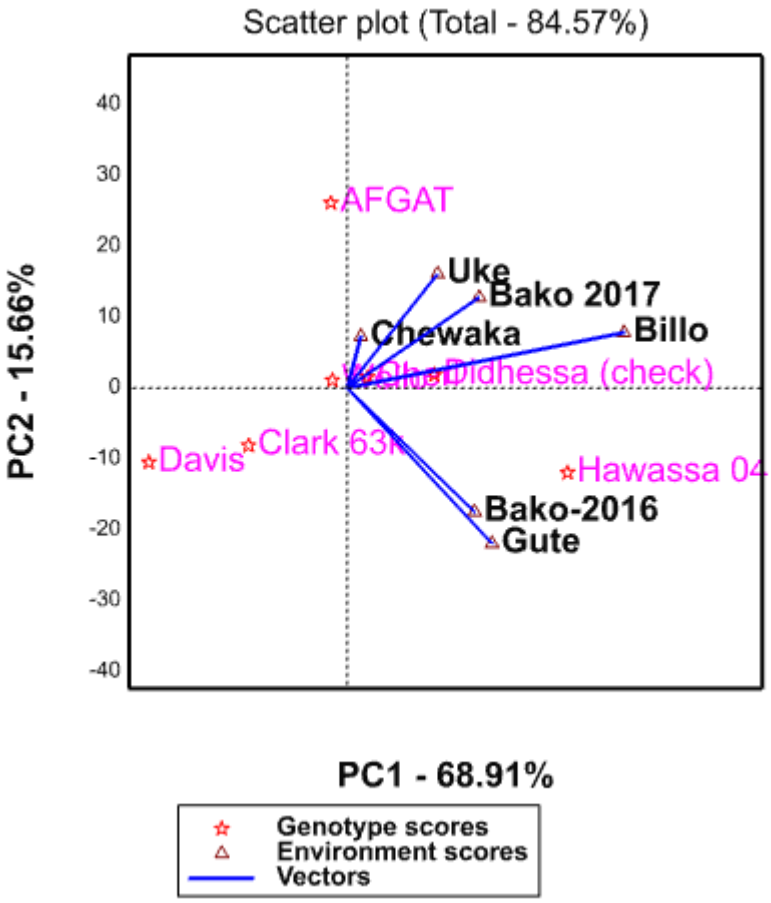

Figure 5. The vector view of GGE biplot which shows the interrelation ships among the test environments and their discriminating ability.

to the ideal genotype and can thus be used as benchmarks for the evaluation of medium maturity groups of soybean genotypes in the western Oromia. Considering simultaneously mean yield and stability, Didhessa and Hawassa-04 were the best soybean varieties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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