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Full Length Research Paper

Characterization of markers linked to resistance motifs against maize lethal necrosis in Tanzanian maize germplasm

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Among the biological constraints facing maize production in Tanzania is a severe occurrence of maize lethal necrosis disease (MLN) raising an urgent need for application of new approaches. A pool of 22 maize genotypes with promising resistance and susceptibility to MLN infection were evaluated by Amplified fragment length polymorphism (AFLP) DNA fingerprinting analysis to detect genetic variation in the selected lines. Eleven AFLP primer combinations were screened and resulted in the identification of 95 polymorphic AFLP allelic fragments. Genetic similarities among the selected Tanzanian maize landraces and other maize lines were estimated by Unweighted Pair Group of Arithmetic Mean (UPGMA) and genotypes were clustered in three primary groups according to their reaction to MLN disease. Promising resistant and tolerant genotypes were grouped in cluster I and susceptible genotypes in clusters II and III. Landraces were grouped according to agro-ecological locations where they were collected. Unambiguous polymorphic AFLP fragments were eluted, purified and sequenced. Sequencing and nucleotide alignment on Basic Local Alignment Search Tool (BLAST) analysis showed similarities of fragments consistent with transcripts involved in disease resistance and stress responses. Further studies will explore the potential application of the identified AFLP markers and their significant association to MLN disease resistance genes in maize.

Key words: *Zea mays,* AFLP, maize lethal necrosis disease, Tanzania.

INTRODUCTION

Maize (*Zea mays* L.) is one of the world's major cereal crops and the third most important crop after wheat and rice (CIMMYT, 1990; Legesse et al., 2006). In recent years, maize has been ranked the first crop in production among other major cereals due to increased global demand for maize both as a major staple food and as an industrial raw material (FAOSTAT, 2016). In Tanzania, maize is the major cereal produced that contributes to

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about 60% of dietary carbohydrates for human consumption and provides more than 50% of utilizable protein for the Tanzanians growing population (Bisanda et al., 1998; Suleiman and Rosentrater, 2015).

Among the biological constraints facing maize production in Tanzania is the occurrence of maize lethal necrosis disease (MLN). MLN is a disease synergism caused by the infection of maize with maize chlorotic mottle virus (MCMV) and any of the potyvirus infecting cereal (Uyemoto et al., 1981). In Africa, MLN was first reported in Kenya in September 2011 and quickly spread to Tanzania in 2012 where it was locally reported as an unknown disease in Mwanza near Lake Victoria area and Arusha (CIMMYT, 2013). This disease has become a major setback in maize growing areas of East Africa (Wangai et al., 2012); hence standing out as the greatest threat to African food security crop (maize). MLN causes serious yield losses of up to 100% depending on the stage of growth of maize plant when it is attacked and particularly when the disease is not effectively controlled (CIMMYT, 2013).

To keep pace with the increased demand of maize due to the expanding population, the development of varieties with enhanced tolerance to biotic and abiotic constraints is thus a significant objective to attain (Boomsma and Vyn, 2008). Effective screening on Tanzanian's maize populations is vital to enhance the identification of genetic resistance for MLN. Currently, there is no published report showing resistance for MLN in Tanzanian maize core germplasms, however, research conducted by International Maize and Wheat Improvement Center (CIMMYT) in Kenya has revealed some promising inbred lines and pre-commercial hybrids with moderate resistance to MLN (CIMMYT, 2013). This underscores an urgent need for application of new approaches such as the use of molecular markers to screening for MLN genetic resistance in Tanzania's maize populations.

To date, a variety of molecular marker techniques have been developed for identifying polymorphisms in plant materials; restriction fragment length polymorphisms (RFLPs) were the first widely used DNA hybridization based molecular markers. Other PCR based techniques include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs) and other emerging techniques (Melchinger, 1990; Stevens, 2008; Wang et al*.,* 2011).

Amplified fragment length polymorphism relies on the use of polymerase chain reaction (PCR) for amplification of DNA. This approach offers several advantages over other DNA markers as it combines the advantages of PCR based technique in terms of efficiency, high throughput and amenability to automation with the specificity and robustness of RFLP based technique (Bhat et al., 2004). AFLP analysis can be applied in any plant species without previous knowledge of DNA sequence (Sigh et al., 2010). Although the technique is laborious and time-consuming, it is highly reliable due to its ability to detect many polymorphic bands in a single lane rather than high levels of polymorphism at each locus as compared to other marker methods such as microsatellite markers (Garcia et al., 2004; He and Prakash, 1997).

Studies that apply molecular markers to access genetic variability and phylogenetic relationships in Tanzanian maize populations are still limited. In this study, we used a powerful molecular technique that does not rely on previously known genes (the AFLP) to screen a diverse set of Tanzania maize germplasm, including landraces and inbred lines. The aim was to identify and characterize genetic markers that may be linked to resistance genes against infection by MLN disease-causing pathogens. A better understanding of resistance to MLN disease in maize and deploying the identified resistance genes in commercial maize varieties could facilitate the genetic control of MLN in Tanzania which would contribute to more practical and effective solutions for small-holder farmers.

MATERIALS AND METHODS

Plant materials

Twenty-two maize genotypes were used as genetic materials in this study that included 12 landraces and 2 lines selected as tolerant and sensitive under artificial MLN disease evaluation at Naivasha MLN screening facility in Kenya by Ritte et al. (2017), 4 CIMMYT maize lines and 4 maize lines of U.S. origin with known MLN disease reaction backgrounds. Landraces and maize lines used to represent Tanzanian maize germplasm were provided by the National Plant Genetic Resources Center (NPGRC) and Selian Agricultural Research Institute (SARI) respectively, both located in Arusha-Tanzania. CIMMYT lines were provided by CIMMYT-Kenya whereas the US maize lines were donated by the University of Nebraska Lincoln and the United States Department of Agriculture (USDA). The US lines were used for preliminary AFLP experiments at Tuskegee University and genomic DNA of these materials was shipped for experiments conducted in Tanzania. Descriptions of these materials are shown in Table 1.

Genomic DNA extraction

Seeds samples of plant materials used in this study were germinated in a screen house at the department of crop science and horticulture, Sokoine University of Agriculture, Morogoro – Tanzania. Young maize leaves were sampled from seedlings of each maize landrace/line at four to five leaf growth stages. Samples were transported on ice to the laboratory and stored at -20°C followed by genomic DNA extraction as described in Egnin et al. (1998). The quality of DNA was assessed on 0.8% agarose gel electrophoresis and the concentration was determined by a known amount of λ DNA as standard. Agarose gel electrophoresis confirmed that the DNA was of high molecular weight with no contaminating RNA or degradation.

Table 1. List of 22 maize germplasm subjected to MLN AFLP screening.

S/N	Genotype ID	Response to MLN	Source
1	CML 494	Promising resistant	CIMMYT
2	CLYN 261	Promising resistant	CIMMYT
3	CLYN 231	Promising resistant	CIMMYT
4	TZA-3567	Tolerant	NPGRC
5	TZA-2793	Tolerant	NPGRC
6	TZA-3585	Tolerant	NPGRC
7	TZA-3543	Tolerant	NPGRC
8	TZA-4505	Tolerant	NPGRC
9	N 218	General Resistance	Nebraska
10	OH 7B	Tolerant	USDA
11	TZA-4320	Moderately Susceptible	NPGRC
12	TZA-5171	Moderately Susceptible	NPGRC
13	TZA-2292	Moderately Susceptible	NPGRC
14	CL-G2620	Susceptible	CIMMYT
15	TZA-5200	Susceptible	NPGRC
16	TZA-4043	Susceptible	NPGRC
17	TUX 5-50-1-3-1-1	Susceptible	SARI
18	KS 03-OB15-111	Very Susceptible	SARI
19	TZA-2264	Very Susceptible	NPGRC
20	TZA-1758	Very Susceptible	NPGRC
21	A635	Very Susceptible	USDA
22	OH 43	Very Susceptible	USDA

AFLP analysis

AFLP analysis procedure was performed with modifications of the protocol of Vos et al. (1995) supplied with the AFLP Analysis System I kit (Invitrogen, USA). About 500 ng of genomic DNA was digested with two restriction enzymes, *Eco*R I and *Mse* I (Invitrogen, USA), at 37°C for 2 h and 30 min followed by incubation at 70°C for 15 min to inactivate the restriction enzymes. *EcoRI* and *MseI* adapters were ligated to the digested fragments at 20°C for 2 h to generate template DNA for amplification. A four-fold dilution was performed on ligated DNA. Pre-selective amplification was performed with 5.5 µl of diluted ligated DNA template, 40 µl of pre-amp primer mix I (*Eco*R I-A/*Mse* I-C), 5 µl of 10X PCR buffer plus $MgCl_2$ and 0.5 µl of *Taq* DNA polymerase (5 U/µl) in 0.2 ml PCR tube. The PCR amplification conditions were set as, 94°C for 30 s, 56°C for 1 min and 72°C for 1 min or 20 cycles in MyCycler Thermal Cycler (*Bio-Rad*, USA). A 4-fold dilution was performed on the pre-amplified reaction in 1X TE buffer and stored at -20°C until ready for use.

For selective amplification, 11 primer combinations (*Eco*R I/*Mse* I) were employed. Each primer pair reaction mix was prepared by combining 5 µl of *Eco*R I primer (27.8 ng/µl) and 45 µl of *Mse* I primer (6.7 ng/µl) to obtain "Mix 1" sufficient for 10 AFLP reactions. Mix 2 reaction mixture enough for 10 AFLP reactions was prepared by pipetting in 1.5 ml Eppendorf tube 79 µl of distilled water, 20 µl of 10X PCR buffer plus $MgCl_2$ [200 mM Tris-HCl (pH 8.4), 15 mM $MgCl_2$, 500 mM KCL], and 1 µl of *Taq* DNA polymerase (5 U/µl). 5 µl of diluted pre-amplified DNA, 5 µl of "Mix 1" and 10 µl of "Mix 2" were combined in a 0.2 ml PCR tube, then subjected to PCR at the following conditions; incubation at 94°C for 30 seconds and one cycle at: 94°C for 30 s; 65°C for 30 ss and 72°C for 1 min, followed by 13 cycles of touchdown PCR where the annealing temperature was lowered by 0.7°C during 12 cycles. This was followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The reaction products were stored at -20°C and used in denaturing polyacrylamide gel electrophoresis (PAGE) analysis.

Denaturing PAGE of amplified AFLP fragments

Denaturing PAGE was performed with modification of the protocol by Summer et al. (2009). An equal volume of 2X TBE-Urea Dye (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA pH 8.0, 10% Glycerol, 0.01% Bromophenol Blue, 0.02% Xylene Cyanol FF, 7 M Urea) was added to each PCR reaction. Samples were denatured by heating at 90°C for 3 min and immediately cooled on ice. PCR products from selective amplification were size separated by horizontal electrophoresis in denatured 6% polyacrylamide gels. Electrophoresis was performed in pre-chilled 1X TBE buffer at constant power (70 V) until Xylene cyanol was about 2-3 cm from the bottom of the gel. After electrophoresis, the gel was post-stained in a 0.5X TBE buffer and ethidium bromide (0.5 µg/ml) with gentle agitation for 25 min, followed by 2 min water rinse, then visualized and images captured by Canon Power Shot A650 (Canon Inc., China) on a UV trans-illuminator (254 nm, with orange filter).

Data analysis

Gel images with amplified fragments were scored in a dominant manner for presence or absence of unambiguous bands as 1 and 0

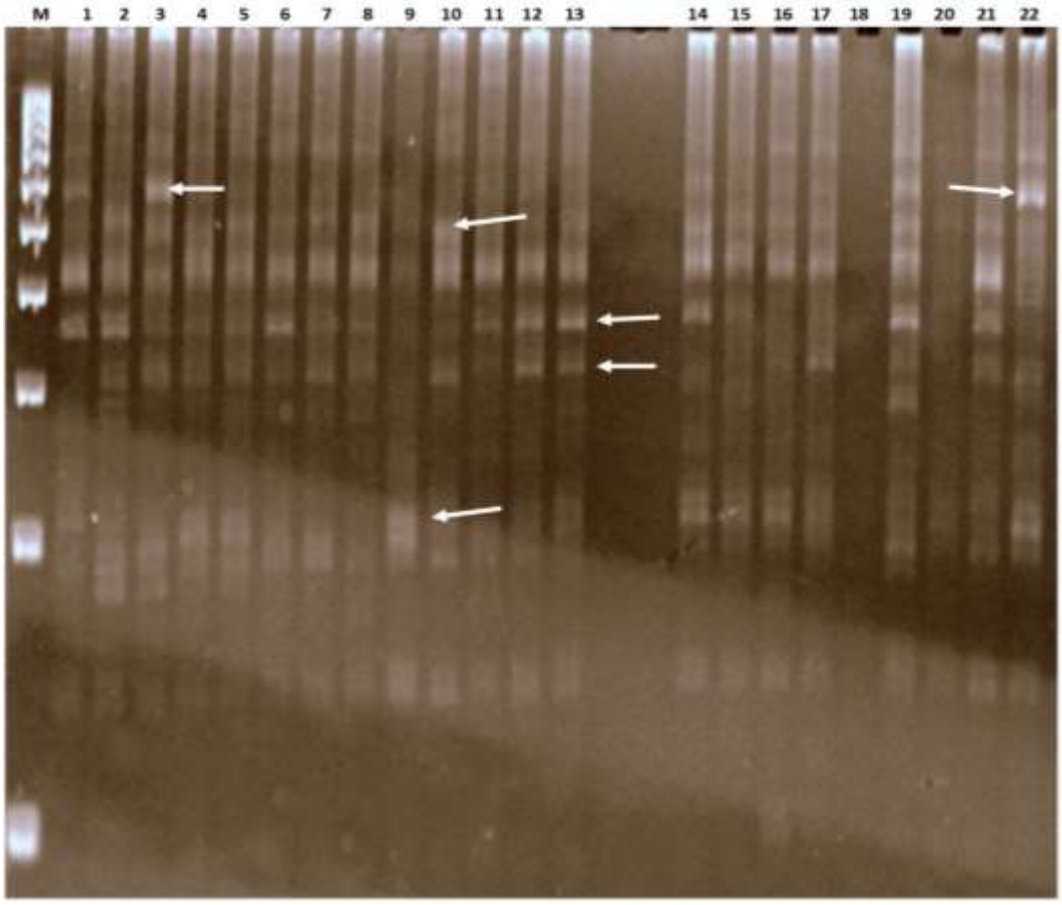

Figure 1. AFLP marker profiles of 22 maize genotypes on 6% polyacrylamide gel. 1, CML494; 2, CLYN261; 3, CLYN231; 4, TZA-3567; 5, TZA-2793; 6, TZA-3585; 7, TZA-3543; 8, TZA-4505; 9, N218; 10, OH7B; 11. TZA-4320; 12, TZA-5171; 13, TZA-2292; 14, CL-G2620; 15, TZA-5200; 16, TZA-4043; 17, TUX5-50-1-3-1-1; 18, KS03-OB15-111; 19, TZA-2264; 20, TZA-1758; 21, A635 and 22. OH43 obtained using primer combination M-CAA/E-ACG. Lane identified by "M" contains 100 bp size DNA ladder ((EZload, Bio Rad®). The arrows indicate polymorphic bands.

respectively to prepare binary matrix. The resulted 1/0 data matrix were exported into a spreadsheet calculated based on the genetic similarity matrix (Nei and Li, 1979) and analyzed using Numerical Taxonomy System (NTSYSpc) software as according to Rohlf (2000). Based on AFLP DNA marker polymorphism data, genetic similarities among the selected Tanzanian maize landraces and other maize lines used in the study were estimated by Unweighted Pair Group of Arithmetic Mean (UPGMA) procedure in cluster analysis and a dendrogram was developed from the similarity matrix (Sneath and Sokal, 1973).

Sequencing and MLN associated AFLP marker development

Unambiguous fragments with strong intensities that were significantly polymorphic in resistant and susceptible genotypes were eluted from gels and purified. Ten microliters of each of eluted fragment were re-amplified with the corresponding primer pairs followed by confirmation on a 2% agarose gel. The confirmed eluted fragments were sent out for sequencing services (Beckman Coulter Genomics Incorporation). Sequence data were uploaded in Bio Edit software version 7.2.5 for sequence editing (Hall, 1999). Edited sequence data were analyzed and compared with sequences of *Zea mays* L. available in the public database using MEGA Software, version 6 by performing nucleotide blast search at the National Center for Bioinformatics (NCBI) Website http://blast.ncbi.nlm.nih.gov/Blast.cgi by using BLASTn program.

RESULTS

AFLP polymorphism

A total of 127 amplified AFLP fragments were revealed among the 22 maize genotypes, 95 of which were polymorphic (Figure 1). The number of amplified AFLP bands ranged from 9 with primer combination (M-CAA/E-AGC) to 17 with primer combination (M-CTG/E-ACA),

Table 2. List of AFLP primer combinations (*Mse* I/*Eco*R I), number of scored AFLP allelic fragments, polymorphic fragments, monomorphic fragments and the percent polymorphism.

S/N	Primer combination	Number of scored allelic fragments	Polymorphic allelic fragments	Monomorphic allelic fragments	Polymorphism (%)
1	M-CTC/E-AAC	13	9	4	69.23
2	M-CTG/E-AAG	10	7	3	70.00
3	M-CTC/E-AAG	11	8	3	72.72
4	M-CAT/E-ACC	10	9	1	90.00
5	M-CAT/E-ACA	12	10	2	83.33
6	M-CTG/E-ACA	17	12	5	70.58
7	M-CAA/E-ACG	13	11	2	84.61
8	M-CAA/E-ACT	10	6	4	60.00
9	M-CTT/E-AGG	12	10	2	83.33
10	M-CTA/E-ACG	10	6	4	60.00
11	M-CAA/E-AGC	9	7	2	77.77
Total		127	95	32	74.68*

*Average polymorphism percentage.

respectively, with sizes ranging from 100 to 800bp. In contrast, the percentage of polymorphism varied from 60% with primer combination (M-CTA/E-ACG) to 90% with primer combination (M-CAT/E-ACC), respectively, and average percentage of polymorphism was 74.7% (Table 2).

Cluster analysis

Binary matrix data that were scored as presence and absence (1/0) of allelic bands were used to construct a dendrogram using unweighted pair group of arithmetic mean (UPGMA) method based on similarity values (Figure 2). The dendrogram revealed three major clusters in which cluster I was further divided into three sub-clusters. The sub-cluster 1.1 included MLN promising resistant CIMMYT lines (CML494, CLYN261, and CLYN231), the sub-cluster 1.2 consisted of the tolerant landraces (TZA-3567, TZA-2793, TZA-3543 and TZA-3585), and the third sub-cluster 1.3 contained tolerant landrace TZA-4505 and the US line N218. Cluster II grouped together the tolerant USDA line OH7B and other MLN moderately susceptible Tanzanian maize landraces. Genotypes TZA-4320, TZA-5171 and TZA-2292 were included in the sub-cluster 2.1. While the sub-cluster 2.2 composed of susceptible CIMMYT line CL-G2620, susceptible landraces TZA-5200, TZA-4043, TZA-1758 and susceptible USDA line A635. The sub-cluster 2.3 grouped together two SARI lines TUX 5-50-1-3-1-1 and KS 03-OB15-111 and the susceptible landrace TZA-2264. The USDA susceptible line OH43 was isolated in cluster III.

Amplicon sequencing

The results of AFLP amplicons sequencing revealed that, out of 63 amplicons sent for sequencing, 32 amplicons were successfully sequenced while 31 amplicons were not. Sequence homology BLAST search at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using BLASTn program revealed different gene functions (Figure 3) and out of 32 amplicons 22 were homologous to *Z. mays* L. reference genome in the database, whereas 10 amplicon sequences were related to other species. Among the 22 amplicons that were in homology with *Z. mays* L. 15 were associated with plant response to biotic and abiotic stresses (Supplementary materials Table 1).

DISCUSSION

The results of this study showed the efficiency of the AFLP technique for determination of molecular polymorphism in maize germplasm. The AFLP primer combination M-CTG/E-ACA yielded the highest number of 17 amplified DNA fragments and M-CAA/E-AGC with the lowest (9). Primer combination M-CAT/E-ACC showed significant molecular polymorphism percent (90%). While more laborious and time-consuming (Garcia et al., 2004), AFLP can lead to the detection of large numbers of bands in a single lane of the AFLP gel, which in turn increases the chance of finding polymorphic markers per lane (He and Prakash, 1997).

These results are in line with those of Maheswaran et al. (1997) who detected a substantial number of

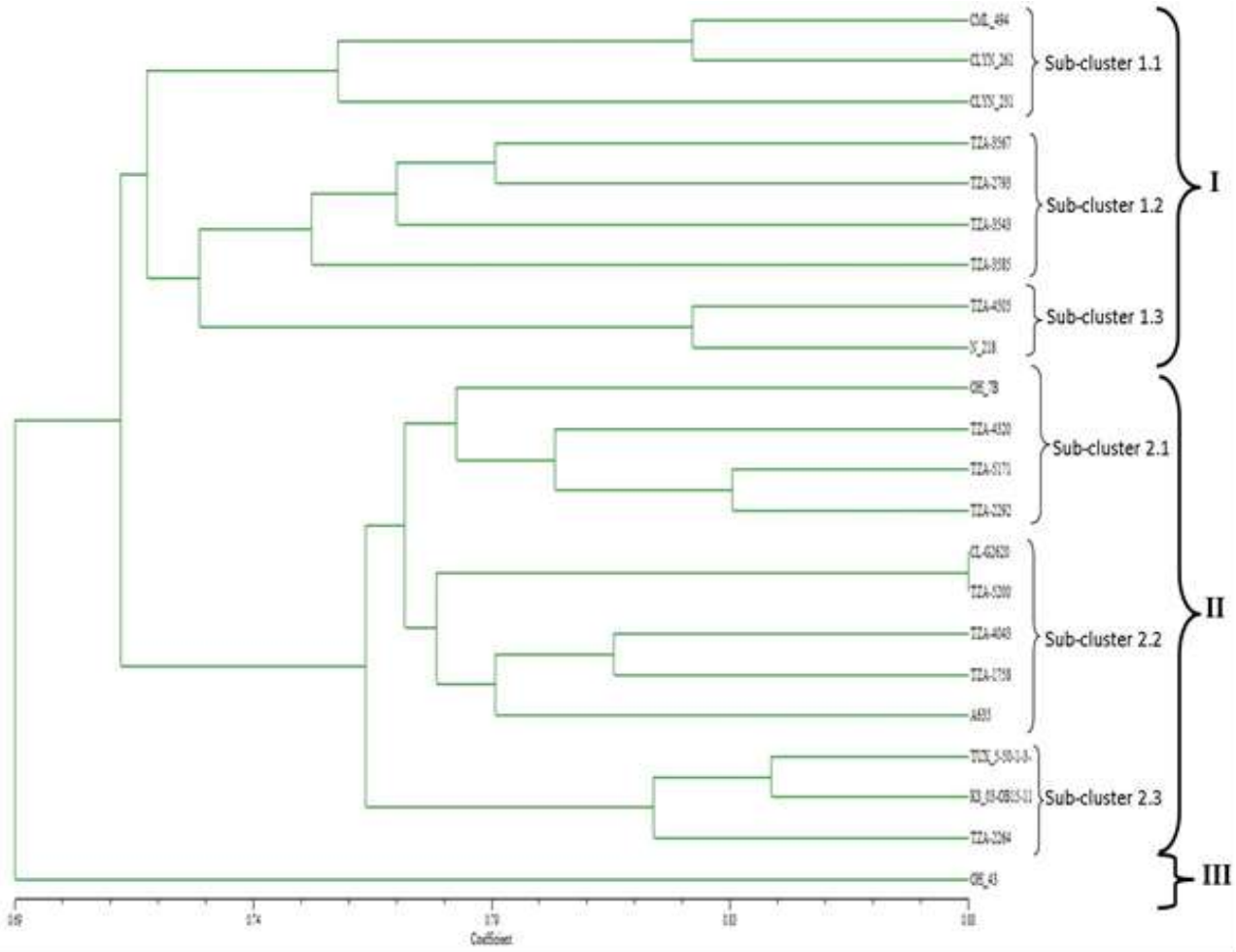

Figure 2. UPGMA dendrogram showing genetic relationships among the 22 maize genotypes generated based on Jaccard's coefficients and AFLP binary matrix data. I, II and III indicate major groups.

polymorphic AFLP bands in studies involving *Oryza sativa*. In this study, low polymorphism was noted on primer pairs M-CTA/E-ACG and M-CAA/E-ACT which attained the polymorphism of 60% respectively. This could be linked to the type of primer combinations used or scoring method applied while conducting the present investigation, as only consistent bands were scored and suspicious bands were not included (Vos et al., 1995). Cluster analysis showed that the genotypes were grouped into three clusters based on their genetic differences, responses to MLN and the geographical origins where landraces were collected. Although the resulted groups were consistent with resistance traits, some mixtures were observed. A similar result has been reported earlier in sorghum accessions and breeding varieties by Uptmoor et al. (2003).

CIMMYT lines (CML494, CLYN261and CLYN231) reported to be MLN promising resistant were pooled to sub-cluster 1.1. Similarly, tolerant landraces TZA-3567, TZA-2793, TZA-3543, and TZA-3585 were as well clustered together in sub-cluster 1.2; landrace TZA-4505 and the resistant US line N218 were grouped in sub-cluster 1.3. In the cluster I, landraces were collected from similar agro-ecological zones in the same region(s), for example, landraces, TZA-3567, and TZA-3543 were collected from Morogoro district in Morogoro region which appears the same for landrace TZA-2793 which was also collected from Kilombero district in Morogoro region as well. Our results suggest that these landraces may have a similar genetic background because farmers usually tend to save and exchange seeds. This may be a reason to presume that similar landrace lines may be known in different vernacular names in the same region. This may apply for landraces TZA-3585 and TZA-4505, which were collected from Mtwara and Ruangwa districts respectively. These two districts are found in the same

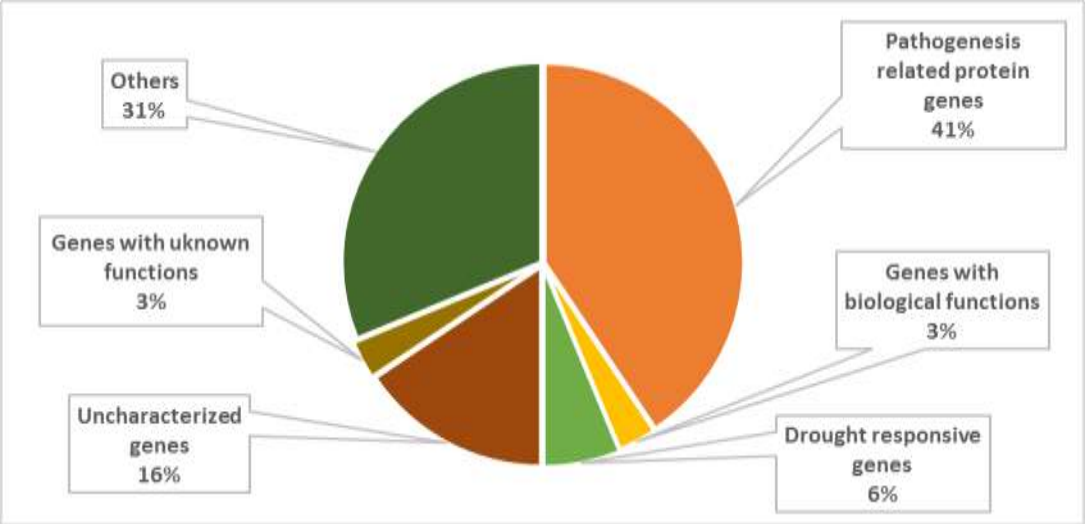

Figure 3. Functional classification of sequenced AFLP markers as they were revealed by the BLASTn program

agro-ecology zone.

The US line OH7B was clustered in cluster II, this maize line is reported to be resistant against MDMV (Roane et al., 1983), a potyvirus also implicated to induce MLN in synergy with MCMV. The reported findings suggest that resistance against any one of the causal viruses could significantly reduce crop damage CIMMYT (2013). Apparently, this line is mentioned to tolerate the incidence of MLN. The tolerance could be due to its ability to resist infection against MDMV.

Other landraces that were identified as susceptible to MLN under artificial inoculation (TZA-4320, TZA-5171, 2292, TZA-5200, TZA-4043, TZA-1758 and TZA_2264) were grouped together in cluster II along with known susceptible CIMMYT line CL-G2620, susceptible US line A635 and two SARI lines TUX 5-50-1-3-1-1 and KS 03-OB15-111 which were also identified as susceptible under artificial inoculation. Line OH43 reported as highly susceptible to MDMV (Roane et al., 1983) was in its own cluster III.

When breeding for disease resistance in plants, two general types of resistance are recognized *viz.* the qualitative and quantitative resistances. The former resistance typically confers a high level of resistance which is usually race-specific and is based on single dominant or recessive genes. In contrast, the quantitative resistance in plants is typically partial and race-nonspecific in phenotype, oligogenic or polygenic in inheritance and is conditioned by additive or partially dominant genes (Wisser et al., 2006). However, it is easier to work with qualitative resistance in crop genetic studies and breeding, quantitative resistance is often the more useful in an agronomic context, due to its generally higher durability (Parlevliet, 2002).

In maize, the majority of disease resistance deployed in elite varieties in the field is quantitative in nature (Wisser *et al.,* 2006). Large number of plant resistance genes have been characterized and efficiently used in many crop breeding programs (Ali and Yan, 2012). A challenge remains to identify new resistance for diseases whose genetic resistance has not been identified and efficiently introgressed in existing germplasm to resist the emerging plant pathogens. For maize lethal necrosis, its genetics and inheritance is reported to be unknown and is expected to be very complicated due to the involvement of two viruses (Manje et al., 2015). However, its genetic resistance is suggested to be poligenically controlled (Nelson et al., 2011).

Genome-wide association analysis studies conducted by Manje et al. (2015) in tropical maize germplasm identified SNP markers that were considered to significantly associate with possible candidate genes for MLN disease resistance. In the same study, B73 maize genome reference sequence was used to identify putative candidate genes based on the SNPs associated with MLN resistance in which a set of putative candidate genes were identified based on their functions. In this work we identified 13 AFLP markers associated with plant defense responsive genes (Supplementary materials Table 1) these markers also had similar functional characteristics (Figure 3) as those reported in Manje et al. (2015). In that regard it is worthwhile to speculate that, the identified AFLP markers may also be associated with resistance of maize against MLN.

The polymorphic bands sequences and nucleotide BLASTn search revealed that the AFLP fragment (244bp)

amplified from the tolerant line OH7B by primer pair M-CAA/E-ACG showed high homology with nucleotide sequences of *Zea mays* presented in the NCBI database. A maximum identity of 97% (E value = 9e-37) was revealed between this polymorphic fragment sequences with *Zea mays* B73 pathogenesis-related protein 2 and GASA-like protein genes. Other AFLP polymorphic fragment sequences which had similar hits with other genes of *Zea mays* B73 were amplified from genotypes TZA-2292 (276 bp), TZA-4320 (279 bp), TZA-3585 (332bp and 386bp), line CLYN231 (276 bp and 281 bp), TZA-5171 (380 bp) and TZA-4043 (355 bp) (Supplementary materials Table 1).

On the other hand, genes of *Zea mays* rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, were also hit by the tested genotypes in this study. The analysis revealed a similarity of 83% (E value= 9e-13) of AFLP marker obtained from line CLYN261which was found to be homologous to *Z. mays* B73 serine/threonine kinase protein and RNA-dependent RNA polymerase (mop1) genes and a homology of 83% (E value = 2e-13) of *Z. mays* putative zinc finger protein of unknown genes (Supplementary materials Table 1). The identified loci such as pathogenesis-related (PR) proteins, rust resistance protein (rp3-1) gene and serine/threonine kinase proteins have been reported to be associated with disease resistance (Bhavani et al., 2013). PR proteins are constituted of highly complex gene families involved in pathogen defense as well as a wide range of normal developmental processes, because of that, they increase the resistance of the plant against pathogenic attack. Such PR proteins play an outstanding role in disease resistance, seed germination and help the plant to adapt to the environmental stress (Adrienne and Barbara, 2006).

The fragment sequences of lines CML494 (330bp) and OH43 (162bp) amplified by primer pairs M-CAA/E-ACG and M-CAA/E-ACG were observed similar with drought responsive lncRNA (complete sequence) from *Zea mays* isolate TCONS_00063399 with E-value 0.064 and 4e-45, respectively. Long non-coding RNAs (lncRNA) are novel molecules with important functions in a wide range of biological processes, which also include developmental regulations and stress responses. A report demonstrated that many lncRNAs participate in responses to a wide variety of biotic and abiotic stresses (Zhang et al., 2014). However, much details on mechanisms involved in these biological processes are not well understood (Kim and Sung, 2012).

The results of this study showed the sequences derived from AFLP polymorphic amplicons associated to disease resistance genes including the pathogenesis-related proteins genes, Serine/threonine kinase protein, rust resistance protein (rp3-1) gene and receptor kinases *Z. mays* putative zinc finger protein genes. Other AFLP amplicons were homologous with plant response to stress such as lncRNA. Therefore, cluster analysis using sequences related to the resistance to pathogens is beneficial towards the identification of resistant genotypes. Further studies will explore the potential application of the identified AFLP markers and their significant association to MLN disease resistance genes in maize.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary materials Table 1. Sequence homology results of sequenced AFLP markers as were revealed by BLASTn program.

S/N	Primer combination and genotype ID	Fragment size (bp)	Function	E-Value	Identity (%)
1	M-CAA/E-ACG **OH7B**	244	*Z. mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds Seq ID:gi/105990542/gb/DQ417752.1	9e-37	97
2	M-CAA/E-ACG **TZA-2292**	276	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds Seq ID:gi/105990542/gb/DQ417752.1	3e-15	88
3	M-CAA/E-ACG **TZA-4320**	279	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds Seq ID:gi/105990542/gb/DQ417752.1	8e-67	86
4	M-CAA/E-ACG **TZA-3585**	386	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds	9e-119	92
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	7e-105	91
5	M-CAA/E-ACG **CLYN231**	276	*Zea mays* B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	2e-45	83

Supplementary materials Table 1. Continue.

S/N	Primer combination and genotype ID	Fragment size (bp)	Function	E-Value	Identity (%)
6	M-CAA/E-ACG TZA-5171	380	*Zea mays* B73 pathogenesis-related protein 2and GASA-like protein genes, complete cds	5e-91	99
7	M-CAA/E-ACG TZA-3585	332	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	1e-55	84
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	1e-54	83
8	M-CAA/E-ACG TZA-4043	355	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	6e-76	95
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	9e-71	93
9	M-CTC-E-AAG CLYN261	144	Zea mays putative zinc finger protein (Z438D03.1), unknown (Z438D03.5), epsilon-COP (Z438D03.6), putative kinase (Z438D03.7), unknown (Z438D03.25) and C1-B73 (Z438D03.27) genes, complete cds	2e-13	83
			Zea mays B73 serine/threonine kinase protein, expressed protein and RNA-dependent RNA polymerase (mop1) genes	9e-13	83

Supplementary materials Table 1. Continue.

S/N	Primer combination and genotype ID	Fragment size (bp)	Function	E-Value	Identity (%)
10	M-CAA/E-ACG CLYN231	281	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	6e-09	95
11	M-CAA/E-ACG TZA-2292	279	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	5e-39	88
12	M-CAA/E-ACG CML494	330	*Zea mays* isolate TCONS_00063399 drought responsive lncRNA, complete sequence	0.064	100
13	M-CAA/E-ACG OH43	162	*Zea mays* isolate TCONS_00063399 drought responsive lncRNA, complete sequence.	4e-45	92
14	M-CTC/E-AAG CLYN231	229	Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	2e-19	86
15	M-CAA/E-ACG CL-G2620	393	Zea mays B73 pathogenesis-related protein 2 and GASA-like protein genes, complete cds	3e-49	84
			Zea mays rust resistance protein rp3-1 (rp3-1) gene, complete cds; and truncated rust resistance protein rp3-2t (rp3-2) gene, complete sequence	5e-40	82

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Agronomic performance of pro vitamin a cassava varieties in three locations in Nigeria

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In an early yellow root cassava study in 2003 to 2004, we investigated agronomic performance of pro Vitamin A (yellow fleshed) cassava genotypes in three locations in Nigeria (Ibadan, Mokwa, and Onne). Twenty-two clones and three checks were evaluated. A randomized complete block design was used with four replications. Characteristics showing significant differences among genotypes across all locations included sprouting, vigor, harvest index, root size, storage root yield, dry matter content and fiber content. Plant height, root mealiness and taste were different in only selected locations. Six clones showed stable performance across locations including IITA TMS I011413, IITA TMS I011442, IITA TMS I011663, IITA TMS I982132, IITA TMS I011277 and IITA TMS I011235. Clones IITA TMS I940330 showed the highest dry matter content of 38%. All clones exhibited good resistant to cassava mosaic disease, cassava bacterial blight, cassava green mite, and cassava anthracnose disease. These genotypes ranged in total carotenoid content from 3.4 to 8.2 µg/g fresh weight. In terms of yield, the best clones were IITA TMS I011368 (26 t/ha), IITA TMS I011663 (22 t/ha) and IITA TMS I982132 (25 t/ha). For gari yield clone IITA, TMS I011649 gave 25%, IITA TMS I940330 gave 23%, and IITA TMS I9001554 gave 23%. They were better than the best check IITA TMS I30572, with 22% gari yield. This study showed the potential for biofortification of cassava as a valid strategy to approach the problem of micronutritient deficiencies of the population in the region where cassava is a staple food.

Key words: cassava varieties, biofortification, pro Vitamin A, Nigeria, agronomic performance.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the third most important source of calories after rice and maize in the tropics. About 60% of the world's cassava production is concentrated in five countries which are Nigeria, Brazil, Thailand, Indonesia and the Democratic Republic of Congo (DRC). Nigeria which is the leading producer with 19% is followed by Brazil with 13 %, Indonesia with 10 %, Thai land with 8% and DRC with 7% while the rest of the world accounts for the remaining 43%. (Ohimain, 2015) The global production of cassava in 2014 was 278.7 million tons with an estimate of 281 million tons for 2015 and 288.4 million tons for 2016 (FAO, 2016). It is a major staple food in Nigeria, consumed daily by more than 100 million people. From available records, Nigeria still stands out as the world's largest producer of cassava with a progressive production pattern that increased from 42.5

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million metric tons in 2010 to 54 million metric tons in 2012 with average production output of 12.2 t/ha in 2010 increased to 14.03t/ha in 2012 (FAOSTAT, 2013). Total area harvested of the crop in 2012 was 3.85 million ha (FAOSTAT, 2013). Over-dependence on cassava-based diets may result in poor health, stunted growth, reduced capacity for physical activity, and in extreme cases, a high incidence of anaemia, corneal blindness, and compromised immunity (Saltzman et al., 2013). However, while the commonly available white cassava can provide most of the body's daily energy requirements, it does not provide sufficient proteins, essential micronutrients–and vitamin A, required for a healthy and productive life. Vitamin A deficiency can impair the body's immunity to infectious diseases and cause eye defect that can lead to partial or complete blindness. Billions of people around the world suffer from hunger and 'hidden hunger' or micronutrient malnutrition. Around 805 million people were considered chronically undernourished over the 2012 to 2014 period (FAO 2014). Nearly one in three Nigerian children under five and one-quarter of all pregnant women in the country are vitamin A deficient (HarvestPlus 2014).

People who do not get enough Vitamin A and micronutrients (zinc and iron) from the foods they eat face severe health complications and even death. Micronutrient malnutrition can lower intelligence quotient (IQ), cause stunting and blindness in children, lower resistance to disease in both children and adults, and increase risks for both mothers and infants during childbirth. Malnutrition is the underlying cause of 45% of child deaths under the age of 5 (WHO, 2015). In 2013, an estimated 161 children under the age of 5 were stunted (below median height for age) and another 51 million were wasted (below median weight for height) (Thompson et al., 2013). This is especially true in regions with prolonged dry seasons that limit production and access to alternative sources of micronutrients such as fresh vegetables (Von Grebmer et al., 2014). Nevertheless, new crosses to select varieties with an even higher content of β-carotene varieties are being generated through recurrent selection breeding scheme (Sánchez et al., 2014).

There is need to work on the Pro Vitamin A cassava clones to understand their performance agronomically. This paper reports on the agronomic performance (diseases and pests, plant height, plant vigor and sprout, root size and numbers root cyanide and dry matter contents, root yield and harvest index) and suitability for quality gari production of adapted beta-carotene rich (Pro-vitamin A) cassava clones in diverse locations (Ibadan, Mokwa, and Onne) in Nigeria.

MATERIALS AND METHODS

The study was conducted at three locations, Mokwa (latitude 9°18'N and longitude 5°04'E; 457 masl; unimodal rainfall pattern with an annual total of 1069 mm, falling between June and October; Radiation is about 450 MJ m^{-2} yr^{-1}. The soil is Alfisols and Ultisols in the southern Guinea savanna zone), Ibadan (latitude 7°31'N and longitude 3°54'E; 150 masl; characterized by a bimodal rainfall also averaging 1300 mm annually, most of which falls between May and October; radiation is about 5285 MJ m^{-2} yr^{-1}. The soil is slightly acidic alfisols;, in the forest savanna transition zone), and Onne (latitude 4° 43'N, longitude 7° 01'E; 10 masl; unimodal rainfall pattern with an annual average of 2400 mm falling between February and December; relative humidity average values range from 78% in February to 89% in July and September; receives an average four hours of direct sunshine daily, reaching 5060 MJ m^{-2} yr^{-1}. The soil is representative of highly leached acid ultisols in the rainforest zone) in Nigeria, following a northern-south gradient of rainfall and vegetation types.

Plant materials and plant establishment

Stem cuttings, each 25 cm long with at least four nodes, were planted in ridges about 50 cm high, 10 m long and spaced 1 m apart in a randomized complete block design with four replications. These sites; Ibadan was planted on the 1st August, 2003, Mokwa on 27th July, 2003, and Onne on 10th August, 2003. The fields were plowed, harrowed and ridged at 1 m apart. 25 clones (23 pro-vitamin A-yellow root and 2 checks- white roots: TME 419 and TME 1) were planted in the basic plot consisted of four rows, making a plot size of 40 m square. The plots were weeded six times after planting. No fertilizers were applied.

One month after planting, data was collected on Cassava Mosaic Diseases (CMD). Cassava Bacterial Blight (CBB) was scored for monthly until 6 months after planting.

Cassava anthracnose disease (CAD) was scored for 6 months after planting and monthly till 9 months after planting. Cassava green mites (CGM) were scored for between January and February. That was when it normally appeared and reached its peak period.

The scale used for scoring was 1 to 5 (1= Zero attack or resistance; 2= little attack or little resistance; 3= medium or moderate resistance; 4= high attack or susceptible and 5= very high attack or highly susceptible).

Number of cassava plants sprouted at 1 MAP

This was counted and scored as number sprouted or germinated over total number planted.

The plant growth vigor at one month after planting was rated visually, per plot basis, using 3 for low vigor; 5 for intermediate vigor and 7 for highly vigor cassava plants.

Plant height was measured, and the mean was calculated

Root size was categorized into small, moderate and large with the scale 3 = small; 5 = moderate and 7 = large.

B- Carotene

Provitamin A carotenoids represent precursors to vitamin A in humans. It was scored at harvest with the use of color chart: 1= white, 2= light cream, 3= cream, 4= light yellow, 5= yellow, 6=yellow deep, 7= orange and 8= pink.

The root cyanide content was estimated by picrate acid method. It was rated on a 1-9 scale based on intensity of red color (higher intensity of =higher HCN content of root sample): 1= <10 HCN; 2= 10-15 HCN; 3 = 15-25 HCN; 4 = 25-40 HCN; 5 = 40-60 HCN; 6 = 60-85 HCN; 7 = 85-115 HCN; 8 = 115-150 HCN and 9 = >150 (Intense red).

Taste of boiled roots

The taste of boiled roots was examined by panel of five people and the conclusion was recorded. The scale used was 1; sweet, 2: bland and 3: bitter.

Dry matter content of the tuberous root is an important character for the acceptance of cassava roots by consumers who boil or process them. Fresh sample of 100 g were taken from each clone in each replicate and dried at 70°C in oven and they were re-weighed after 72 h of drying and have attained constant drying. The dried sample was weighed, and root dry matter content percentage was calculated as the ratio between fresh weight (FW) and dry weight (DW) that is DM (per cent) = (DW/FW) × 100.

Fresh root yield

All the underground roots per plot were weighed in kilograms (kg) and converted to tones per hectare (t/ha).

Gari yield

Gari yield is the weight of gari (a dried granule gotten from ten kilograms of fresh cassava roots of each clone, after peeled, grated, fermented, dewatered, fried and sieved) measured in kilograms.

Harvest index was calculated by (root weight / root weight + shoot weight) × 100.

Statistical analysis

The collected data were subjected to analysis of variance (ANOVA) using General Linear Model (GLM) procedure in statistical analysis system (SAS, 1996) to test for the treatment of effect and significant interaction of the variables considered. The results of the different experiments were subjected to combined analysis of variance to examine G × E effect and standard errors were calculated for each trait.

RESULTS AND DISCUSSION

Locations accounted for most of the G × E interaction significance (p=0.001) which reflects the differences in soil types in which the clones were grown. This suggested that for the evaluation of cassava clones, it might be more appropriate to test genotypes over space rather than over time. The soil variation observed justified the effects of blocks on the performance of most traits, showing the importance of conducting and blocking genotype trials across various environments for selecting genotypes presenting general and/or specific adaptation to those environments. The findings on G × E interaction agreed with the findings of Tumuhimbise et al. (2015) showing that G × E interaction analysis is important for identifying genotypes with adequate adaptation to target environments.

Plant height

There were significant differences among the clones in Onne with CV of 17%, while in Ibadan and Mokwa a non-significant difference was observed (Table 1).

Vigor

The effect of G × E on vigor was very significant (Table 1) with CV of 17.90%. The effect was not significant only in Ibadan. This shows that vigor is a stable trait and not affected by the environment.

Fresh tuber yield

Tuber yield vary significantly among the clones and G × E analysis (p = 0.001) was also significant (Table 1). There was wide spread of the difference in the mean of the yield among the genotypes and across the locations. Osekita et al. (2014) also had a wide spread of difference in the mean of the yield among the genotypes and across the locations in his findings. The yield could be considered stable in Mokwa and Onne with a high yield of about 20 t/ha. In terms of fresh root yield, the best clones were 01/1368 (26 t/ha), 98/2132 and 01/1663 (25 t/ha). Clones 01/1412, 01/1115, 01/2135, 01/1610, 01/1649. 95/0379 gave between 21 and 22 t/ha.

Dry matter

Dry matter content is a very important trait for acceptability of the cassava by consumers. It was significant in Ibadan, with CV of 11.17%. In Onne, it was not significant. However, in combined analysis it was significant at a probability level of 0.001 with a CV of 12.88%. The significant effect of the clone on cassava dry matter agrees with the findings of Athanase et al. (2017)

The dry matter percentage mean ranged from 27.5 to 38 across the locations (Table 3). This shows high dry matter percentage. Edoh et al. (2018 also reported higher dry matter percentage of 33.5% in one of her findings.

Beta- carotenoid

Beta- carotenoid content ranged from 1.0 to 7.0 µg/g fresh weight Table 3.

Locations accounted for most of the G × E interaction significance (p=0.001) which reflects the differences in soil types in which the clones were grown. This suggested that for the evaluation of cassava clones, it might be more appropriate to test genotypes over space rather than over time. This experiment was able to identify stable clones and high beta- carotene across locations among the clones used: 01/1413, 01/1442, 01/1663, 98/2132, 01/1277, and 01/1235. A few

Table 1. Mean square from analysis of variance showing various agronomic traits of 25 yellow root cassava genotypes evaluated for multilocational traits at Ibadan, Mokwa and Onne during cropping season.

Trait	Mean	CV%	MS btw clone df = 24	Sig	Mean	CV%	MS btw clone df =24	Sig. level	Mean	CV%	MS btw clone df =24	Sig. level
F.Yield	15.20	35.8	86.83	**	15.10	42	258.4	***	24.1	25	116	***
Sprout	95.67	7.79	337.1	*	18.16	66	221.6	ns	93.9	9.4	205	ns
Vigor	6.25	10.66	1.78	ns	5.64	18	1.88	**	4.4	21	1.7	*
Leaf area	11697	228.5	6736	ns	15222	58.4	54428	ns	26598	66.4	46798	ns
Plant height	63.26	41.28	428	ns	59.70	30	277	ns	117	17	695	*
Dry matter	35.26	11.17	37.40	ns	34.5	15	34.56	*	28	18	35	ns
Mealy	1.05	119.7	2.5	ns	2.64	14	0.53	***	0.5	131	0.82	ns
Cyanide	5.68	20.47	3.22	**	5.68	20	3.22	***	4.2	20	4.21	*
B-carotene	5.45	9.88	10.55	**	5.43	9	11.61	**	4.6	19	3.23	*
R.Size	6.68	12.06	0.57	ns	6.64	11	1.38	**	6.6	13	2.6	***
Taste	1.65	24.63	0.44	**	1.68	20	1.06	*	1.8	27	0.3	ns
Gari	20.65	15.23	20.68	**	19.65	24	15.65	*	19	9.8	19	***

*, ** &*** indicate 0.05, 0.01 and 0.001 levels of significance and ns means not significance.

genotypes were high up to7 when a color chart that ranges from 1 to 8 was used. Olapeju et al. (2013) also reported higher carotene concentration in some of the varieties. Tuber yield, dry matter content, root size, fiber content, harvest index, sprouting and vigor of the varieties evaluated were all significant among the clones in the combined analysis (Table 2). Taste color of un-expanded leaves, height at branching, leaf area, and internode length were not significant.

Overall dry matter content showed that clone 94/0330 (yellow root) had the highest dry matter (38%), which was better than the best check 30572 (36%), followed by the clones 01/1115, 01/1413, 01/1663, with values ranged from 30 to 35%. However, in Ibadan plant height, vigor, mealiness and root size were not significant (Table 1). In Mokwa; sprouting and plant height were the only traits that were not significant (Table 1). In Onne dry matter, sprouting, mealiness and aste were not significant (Table 1).

The significant effect of Cassava Mossaic disease (CMD) indicated that genotypes respond differently to CMD in various environments, explaining the need for specific adaptation analysis for the trait, as reported by Athanase et al. (2017). In terms of disease and Pest resistance, all the clones evaluated were resistant to CMD, CBB and CGM vector infection and to the spread of the pathogen within the plant and across the locations (Table 4).

Conclusion

In terms of yield, the best clones were 01/1368 (26 t/ha), 98/2132 and 01/1663 (25 t/ha). Clones 01/1412, 01/1115, 01/2135, 01/1610, 01/1649. 95/0379 gave between 21 and 22 t/ha. In terms of the cyanide level, clones 01/1442, 01/1413, 01/1115, and 01/1663 were very low, clones 01/1224, 01/1235, 01/1371, 95/0379, 98/2132, 94/0006, 01/1662, and 01/1412 were moderate. None of them was high in cyanide level. Most of the root sizes were large and some were moderate while none were small among the clones evaluated. In terms of harvest index, clone 01/1115 had the highest index of about 120% of the total yield. Clones 98/2132 (60%), 01/1235(59%), 01/1412 (58%), and 95/0379 (55%), were acceptable. Clones 01/1115 and 98/2132 were better than the best check (91/02324) with a harvest index of 60%. Clones 01/1235, 01/1412, 98/2132, 01/1115, 95/0379, 94/0006, and 01/1649 were better than the most popular check 30572 (55%). Four of the clones used in this experiment were already released varieties in Nigeria. Three of them (01/1368, 01/1412 and 01/1371) were released in the year 2011 while, one (98/2132) was released in the

Table 2. Mean performance of G × E of 25 beta-carotene cassava evaluated for multilocational trails at Ibadan, Mokwa, onne for agronomic traits effect during 2004 cropping season.

Traits	Mean	CV%	MS btw clone df = 24	Sig.level
F.Yield	19.63	31.28	125	***
Sprout	1.92	11.19	0.06	***
Vigor	09	17.90	3.03	***
H.I	0.51	14.55	0.02	***
Root size	6.63	12.49	1.74	***
Fiber	2.37	9.36	0.16	***
Dry matter	32.91	12.88	50.1	***
Taste	69	25.09	0.24	Ns
Leaf shape	13	12.03	1.17	***
C. of unexp Leaf	65	34.65	11.47	Ns
Pub.of young leaf	2.78	45.77	5.50	**
Petiole length	12.33	34.36	35.21	*
Petiole C	3.5	29.37	6.5	***
Flowering	1.7	35.79	0.31	**
Fruit	1.2	89.35	0.37	*
Height at B	26.91	81.50	799.9	ns
Stem/plant	1.19	27.31	0.44	**
Internode L	1.21	18.87	0.05	Ns
Stem color	2.5	23.85	1.26	**
D. of Anthocyanine pigment	1.81	60.62	6.64	*

*, ** &*** indicate 0.05, 0.01 and 0.001 levels of significance and ns means not significance.

Table 3. Mean performance of G × E beta-carotene in cassava genotypes evaluated for multilocational trial at Ibadan, Mokwa, and onne for agronomic traits effect during 2004 cropping season.

Clones	F. Yield	H.I.	DM	B-Carotene	Cyanide	Root Size	Taste	Gari Yield/50 kg	Gari yield
01/1115	21.21	1.2	29.53	7.00	3.50	7	1.8	8.0	16
01/1224	17.96	0.5	34.17	6.75	4.75	7	1.5	10.5	21
01/1235	21.40	0.59	28.99	6.00	4.50	7	1.3	6.0	12
01/1273	16.21	0.5	28.00	6.75	4.75	7	1.7	7.2	14.4
01/1277	16.20	0.5	34.12	6.50	4.50	6.3	1.7	7.5	15
01/1331	9.28	0.36	30.84	6.25	5.50	5.3	2	7.3	14.6
01/1335	18.50	0.53	31.88	7.00	4.25	7	1.5	8.5	17
01/1368	26.13	0.05	30.12	6.00	5.25	7	1.8	7	14
01/1371	17.86	0.52	30.04	6.75	4.50	6.7	2	6.5	13
01/1412	21.96	0.58	28.08	6.50	4.50	7	1.8	10	20
01/1413	19.06	0.52	28.97	7.00	3.25	5.8	1.5	2.0	4
01/1442	16.58	0.53	30.44	6.25	3.00	6.3	1.7	7.0	14
01/1610	20.61	0.52	27.52	6.75	4.75	6.7	1.8	10	20
01/1646	18.10	0.45	31.58	5.50	4.00	6.3	1.7	7.7	15.4
01/1649	20.88	0.56	32.19	6.25	4.25	7	1.5	12.5	25
01/1662	16.38	0.46	29.90	5.50	4.00	6.5	1.8	10.6	21.2
01/1663	24.54	0.54	29.02	7.00	3.50	7	2	10	20
30572	26.83	0.55	37.18	1.00	3.75	6.7	1.5	11	22
90/01554	19.95	0.49	34.97	4.25	4.25	7	1.8	11.5	23
91/02324	24.66	0.60	35.30	1.00	3.25	7	1.3	8.2	16.4
94/0006	20.84	0.60	35.19	4.25	4.25	6.7	1.7	7.4	14.8
94/0330	13.89	0.41	38.35	4.50	5.25	6.3	1.7	11.5	23

Table 3. Contd.

95/0379	20.81	0.55	29.59	6.00	4.00	6.7	1.8	6.5	13
98/2132	25.02	0.69	35.78	6.00	4.25	7	2	7.0	14
TME 1	18.01	0.54	33.62	1.00	3.25	6.8	1.5	8.2	16.4
G.MEAN	19.73	0.55	31.81	5.51	4.23	6.68	1.7	8.4	
STDEV	3.91	0.15	3.09	1.88	0.67	0.43	0.20	2.23	
Stderr	0.01	0.03	0.62	0.38	0.13	0.12	0.06	0.64	
CV%	31.28	0.60	12.88	7.52	2.69	12.49	12.88	9.78	
F-Ratio	***	***	***	***	***	***	ns	ns	

*, ** & *** indicate 0.05, 0.01 and 0.001 levels of significance and ns means not significance. H.I = Harvest Index; DM = Matter; F. Yield; R. Size = Root size.

Table 4. Mean from analysis of variance showing the reaction of 25 yellow root of Cassava genotype to CMD, CBB, CAD and CGM severity and incident at Ibadan, Mokwa and Onne.

Traits	Range	Ibadan			Mokwa			Onne		
		Mean	CV (%)	Sig	Mean	CV (%)	Sig	Mean	CV (%)	Sig.
CMD(s)	1−5	1.7	28.8	**	1.42	14.51	**	1.7	15.1	**
CBB(s)	1−5	1.8	22.03	*	2.45	14.31	***	1.49	7.14	*
CMD(I)	1−5	2.02	30.36	Ns	1.11	58.55	***	1.19	59.2	***
CBB(I)	1−5	1.18	39.74	Ns	1.9	18.49	**	1.07	93.58	**
CGM	1−5	4.04	24.6	**	2.9	15.05	**	2.34	6.57	Ns
CAD	1−5	18.8	26.64	Ns	1.89	25.6	ns	1.84	18.29	**

*,**,*** indicate 0.05, 0.01 and 0.001 levels of significance and ns means not significant. CMD = Cassava Mosaic Disease; CBB = Cassava Bacterial Blight; CAD = Cassava Anthracnose Disease; CGM = Cassava Green Mite; S = Severity; I = Incident. 1, zero attack or resistance; 2, little attack or little resistance; 3, medium or moderate resistance; 4, high attack or susceptible; 5: very high attack or highly susceptible.

year 2012. All clones were resistant to CMD, CGM and CAD vector infections across the locations. Clones 01/1115, 01/1413, 01/1663, and 01/1335 had higher beta-carotene content than others. For gari yield, clone 01/1649 gave 25%, 94/0330 gave 23%, 90/01554 gave 23%. They were better than the best check 30572, with 22% gari yield.

This study showed the understanding of agronomic performance of the early potential for biofortification of cassava as valid strategy to approach the problem of micronutrient deficiencies of the population in the region where cassava is a staple food.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of native mycorrhizal fungi inoculants on the growth and phosphorus uptake of tree legumes: *Erythryna brucei and Millettia ferruginea*

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The inoculation study was conducted in the greenhouse to investigate the effect of phosphorus (P) concentrations on growth and arbuscular mycorrhizal fungi (AMF) colonization of multipurpose tree legumes *Erythrina brucei and Millettia ferruginea*. Plant growth parameters (shoot length, dry weight) and P uptake increased significantly after inoculations with AM fungi, *Rhizophagus clarus, Rhizophagus intraradices* and the mixed species. Results on effect of P application on total Mycorrhizal Dependency(MD) of studied tree species showed that maximum MD values were recorded for *R. clarus* (34.87%) in *M. ferruginea* and (26.19%) in *E. brucei* respectively. For the mixed species was recorded, the next highest MD values 26% in *M. ferruginea* and 16.67% in *E. brucei.* The least MD values were recorded for treatments with *Rh. intraradices* in both trees under study. The optimum P concentrations for maximum benefits from the AM symbiosis in aforementioned tree species varied from 0.005 to 0.02 mg g^{-1} and corresponding peaks of arbuscules, vesicles, percent root colonization, and spore count per 50 cm^3 sand were noticed at similar concentrations. Thus, the results showed that the recorded plant growth peaks were due to AM colonization of the tree seedlings. Therefore, inoculating plants with a suitable AM inoculant could result in a benefit comparable to high P input and lead to a significant saving of inorganic P fertilizer.

Key words: Agroforestry, trees, *root* colonization, spore density.

INTRODUCTION

*Erythrina brucei (*Schweinf) and *Millettia ferruginea* (Hochst) Baker from the family *Fabacae* (Leguminosae) are the most common shade trees in agroforestry systems of Gedeo and Sidama of Southern Ethiopia. In addition to Gedeo and Sidama, *E. brucei* grows naturally in open places and along edges of upland forests or woodlands in Wello, Gojjam, Shewa, Bale and Hararge, and Keffa agroforestry systems at altitudes between 1400 and 2600 masl. It flowers from November up to January and at times of fruiting most of its leaves shed adding to

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the organic fertility of the soil.

M. ferruginea is also one of the most valuable multipurpose tree species of Ethiopia (Legesse, 1995; Tadesse et al., 2000). It is used to improve soil physical and chemical properties in agricultural activities, as fodder for ruminants, as a shade tree, building materials, and used as medicine (Legesse, 1995, 2002; Tadesse et al., 2000).

M. ferruginea commonly occurs between 1100–2500 m above sea level and is characterized as a component of upland forest (Thulin, 1989). *E. brucei* and *M. ferruginea* (Hochst) Baker (Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1998, 2011; 2002) are good biological nitrogen fixers and can be used as organic sources of nitrogen in agroforestry systems.

These multipurpose shade trees play a vital role in the rural economy of the region. In order to meet the future demand of these shade trees, their growth and productivity has to be hastened from the nursery stage onwards and their requirements for major fertilizers like Phosphorus should be known. Furthermore inappropriate and untimely application of fertilizer in agricultural fields generated several environmental and soil problems (Tilman et al., 2002; Foley et al., 2005).

The perceived need to seek alternatives to current agricultural practices has resulted in an enhanced interest in agroforestry systems (Ingleby et al., 2007), which can conserve resources, improve environmental quality, rehabilitate degraded lands, and provide multiple outputs to meet the daily demands of the rural population (Pande and Tarafdar, 2004; Muleta et al., 2008).

Agroforestry, a land use system/technology in which trees are deliberately planted on the same unit of land with agricultural crops, has been recognized as one of the most promising strategy for rehabilitating degraded areas. Arbuscular mycorrhizal (AM) fungi can rehabilitate degraded lands subjected to agroforestry systems (Mutuo et al., 2005; Cardoso and Kuyper, 2006). The common mycorrhizal network may further enhance the benefits of agroforestry through vertical niche expansion of AMF (Cavagnaro et al., 2005; Theuerl and Buscot, 2010).

The need to increase food, fibre, and fuel wood production to keep pace with the fast-growing population is crucial (Wrage et al., 2010). The low biomass production of agroforestry tree species such as in degraded areas can, therefore, be circumvented by the use of AM fungi (Shukla et al., 2009).

Phosphorus (P) is an essential nutrient for plant growth (Schroeder and Janos, 2005) and it is taken up by plants as phosphate (Landis and Fraser, 2008), which is unevenly distributed and relatively immobile in soils (Baird et al., 2010; Gianinazzi et al., 2010). The key function of AM fungi is the exploration of the soil beyond the range of roots for better plant growth and nutrition (Oehl et al., 2002; van der Heijden et al., 2006; Yadav et al., 2013a) AMF has the potential to make cultivation successful at a lower soil P level through more effective exploitation of the P sources (Jakobsen et al., 2005; Ma and Rengel, 2008).

The P level has been shown to significantly influence AM colonization (Covacevich et al., 2007). Addition of P fertilizers above optimum can delay or decrease colonization of roots (de Miranda et al., 1989; Hinsinger, 2001) and reduce chlamydospore production by the fungus (de Miranda and Harris, 1994). Agroforestry is not only concerned with beneficial effect of one component on another, but also involves the manipulations of negative effects to minimize their influence on the productivity of the overall system. At the tree-crop interface of an agroforestry system, trees and crops compete inevitably for light, water, and nutrients and AMF play an important role in P uptake. Therefore, the present study was conducted to identify the effect of AMF inoculation and application of different rates of phosphorus on growth and P uptake of leguminous tree species *E. brucei and M. ferruginea* that grow in Sidama and Gedeo agroforestry.

MATERIALS AND METHODS

In this study, seeds of the selected shade trees in Sidama and Gedeo agroforestry were used. Three native species of AM fungi isolated and purified from the rhizosphere of trees and crops from Sidama agroforestry were used as AM inoculants. Taxonomic identification of spores was checked to be matched with the description provided by the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM, 2006). Inoculum used in this study was consisted of soil along with chopped root bits of *Sorghum bicolor*, spores, and extrametrical mycelia from trap culture pots.

To study the effect of P concentrations on tree growth and P uptake after inoculation with AMF, separate experiments were carried out for the two multipurpose shade trees in the agroforestry. The trials consisted of six P concentrations (0, 0.005, 0.01, 0.02, 0.05, and 0.1 mg/g) and three mycorrhizal treatments (*Rhizophagus clarus, Rhizophagus intraradices* and mixture of the two) and un-inoculated plants (control). Thus, a total of 24 treatments were carried out per each plant species, and each treatment was replicated three times. Seeds were surface sterilized with Sodium hypochlorite, washed several (five to six) times with sterilized distilled water and germinated on sterilized river sand at 30°C. In this study plastic pots filled with 2 kg sterile sand were used.

At the time of sowing, 50 g of mycorrhizal inocula was applied to the hole where pre-germinated seedlings were individually transplanted. Phosphorus was applied to the pots at 0, 0.005, 0.01, 0.02, 0.05, and 0.1 mg/g as KH_2PO_4. Plants were grown under greenhouse conditions and watered daily. One seedling was maintained per pot and half-strength Hoagland's solution in deionized water was applied at weekly intervals. The composition of the Hoagland's solution was (0.51 g/L KNO_3, 0.246 g/L $Ca(NO_3)_2$, 0.245 g/L $MgSO_4.7H_2O$, 1.43 g/L H_3BO_3, 0.91 g/L $MnCl_2.7H_2O$, 0.11 g/L $ZnSO_4.5H_2O$, 0.04 g/L $CuSO_4.5H_2O$, and 0.04 g/L $H_2MoO_4.H_2O$). To reduce the risks of cross contamination, pots were kept on separate benches, with a space of 40 cm between each treatment.

Seedlings were harvested after three months and analyzed for shoot length and dry weight by standard methods. Phosphorus uptake was recorded using Molybdenum blue method according to Jackson (1973). Mycorrhizal dependency was calculated according to Plenchette et al. (1983): [(M-NM)/M] × 100, where: M is the total dry biomass of mycorrhizal plant; NM is the total dry biomass of

non-mycorrhizal plant.

To study the effect of P application on AM colonization of the two trees, aforesaid treatments (24) were replicated four times and six plants were maintained per replicate/pot. Two plants per pot were harvested after 1, 2, and 3 months of sowing, for observations. Formation of arbuscules and vesicles were monitored and then, was calculated the colonization index and spore count per 50 cm^3 sand. Fine roots were cleared with 10% KOH and stained with acid fuchsin (0.01% in lactoglycerol) as reported by Phillips and Hayman (1970) and then was recorded colonization rates of arbuscules and vesicles. Colonization percentage was determined by gridline intersection method of Giovannetti and Mosse (1980). Sporocarp and spores were isolated according to Gerdemann and Nicolson (1963) and counted.

Statistical analysis

All the data on plant growth were subjected to a one-way analysis of variance for testing the effects of AM inoculation and P application, and their interactions. The means were compared and ranked using Duncan's Multiple range test (P<0.05). The mean of experiments were analyzed statistically using a general linear model for analysis of variance of completely randomized designs. Analysis of variance (ANOVA) and correlation analysis were carried out with the SPSS software package (version 20.0). (SAS, 1982).

RESULTS

Plant growth, shoot dry biomass and P uptake

The results on effect of AM inoculation (*Rh. clarus, Rh. intraracices and* the mixed species) and P (0, 0.005, 0.01, 0.02, 0.05, and 0.1 mg g^{-1}) application on growth and P uptake by *E. brucei* and *M. ferruginea are* presented in Figure 1. Most of the peaks of shoot length, and dry weight, by these trees occurred from 0.005 to 0.02 mg g^{-1}. In un-inoculated plants, such peaks were inclined towards increasing P concentration. For the three AM fungi studied, these peaks indicate that the optimum P concentrations for maximum benefits from the AM symbiosis in plant species lied mostly from 0.005 to 0.02 mg g^{-1} P concentration. For shoot length the optimum P concentration for most effective AM inoculants, *Rh. clarus*, *Rh. intraradices* and the mixed species in *E. brucei* and *M. ferruginea* was 0.02 mg P g^{-1}. In both *E. brucei* and *M. ferruginea* inoculated with the three AM species, plant height increased with increasing P concentration until P=0.02 mg g^{-1} and shoot dry weight has increased with increasing P concentration until P=0.01 mg/g. However, the increase both in shoot length and dry weight of both *E. brucei* and *M. ferruginea* has decreased with increasing P concentration above p= 0.02 and P=0.01 mg g^{-1} respectively. Thus, the two tree species inoculated with the three AM species has positively reacted with increasing P concentration and inoculating above-mentioned trees, with a suitable AM inoculant (at lower P concentration) can be effective as high inputs of recommended P fertilizers.

Therefore, the optimum P concentration for the two selected agroforestry shade trees studied with different AM fungi for maximum benefit from the symbiosis was low (0.005–0.02 mg P g^{-1} substrate). Since different AM fungi can transport different amounts of P to plants, their effects on plant growth can also be different. Despite this fact however, in the current study the two species from *Glomeromycota* and the mixture of the two has produced similar results in the green house as compared to the un-inoculated which has been given similar P concentration with other treatments. In both trees studied, AM inoculants used; *Rh. clarus, Rh. intraradices* and the mixed species has significantly increased shoot length, dry weight, and P uptake at P<0.05% level.

Mycorhizal dependency (MD) of seedlings of the trees

Total results on mycorrhizal dependency of *E. brucei and M. ferruginea* seedlings are presented in Table 1. In both trees, the three AM inoculants: *Rh. clarus, Rh. intraradices* and the mixed species significantly (p<0.005) increased shoot length and total shoot dry biomass. Maximum MD values were recorded for *Rh. clarus* (34.87%) in *M. ferruginea* and (26.19%) in *E. brucei* respectively. For the mixed species was recorded, the next highest MD values 26% in *M. ferruginea* and 16.67% in *E. brucei.* The least MD values were recorded for treatments with *Rh. intraradices* in both trees under test.

Effect on AMF structural colonization and spore density

Arbuscular mycorrhizal fungi structural colonization (Arbuscules & Vesicles) of the trees after inoculation with *Rh. clarus, Rh. intraracices* and the mixed species are presented in Table 2. In the current study, formation of arbuscules by *Rh. clarus and Rh. intraradices* and the mixed species was more favored at lower P concentrations (0.05 to 0.02 mg P g^{-1} substrate). However, there were also some rates of colonization below and above 0.05 and 0.02 mg g-1 p concentration in all inoculated tree species (Table 2). The result also indicates that arbuscule formation was the earlier during the 1st month of inoculation and that of formation of vesicles was intensive during the 2nd and 3rd months of the inoculation.

Finally, the tree species inoculated with AM fungi showed mycorrhizal colonization that was characterized by the presence of arbuscules and vesicles (Table 2). However, mycorrhizal colonization, arbuscule and vesicle formation decreased significantly with the increase in P concentration. Also similar trend was observed with mycorrhizal spore number (Table 3), and positive correlation was recorded between mycorrhizal spore number and percentage root colonization.

Maximum root colonization and spore count per 50 cm^3

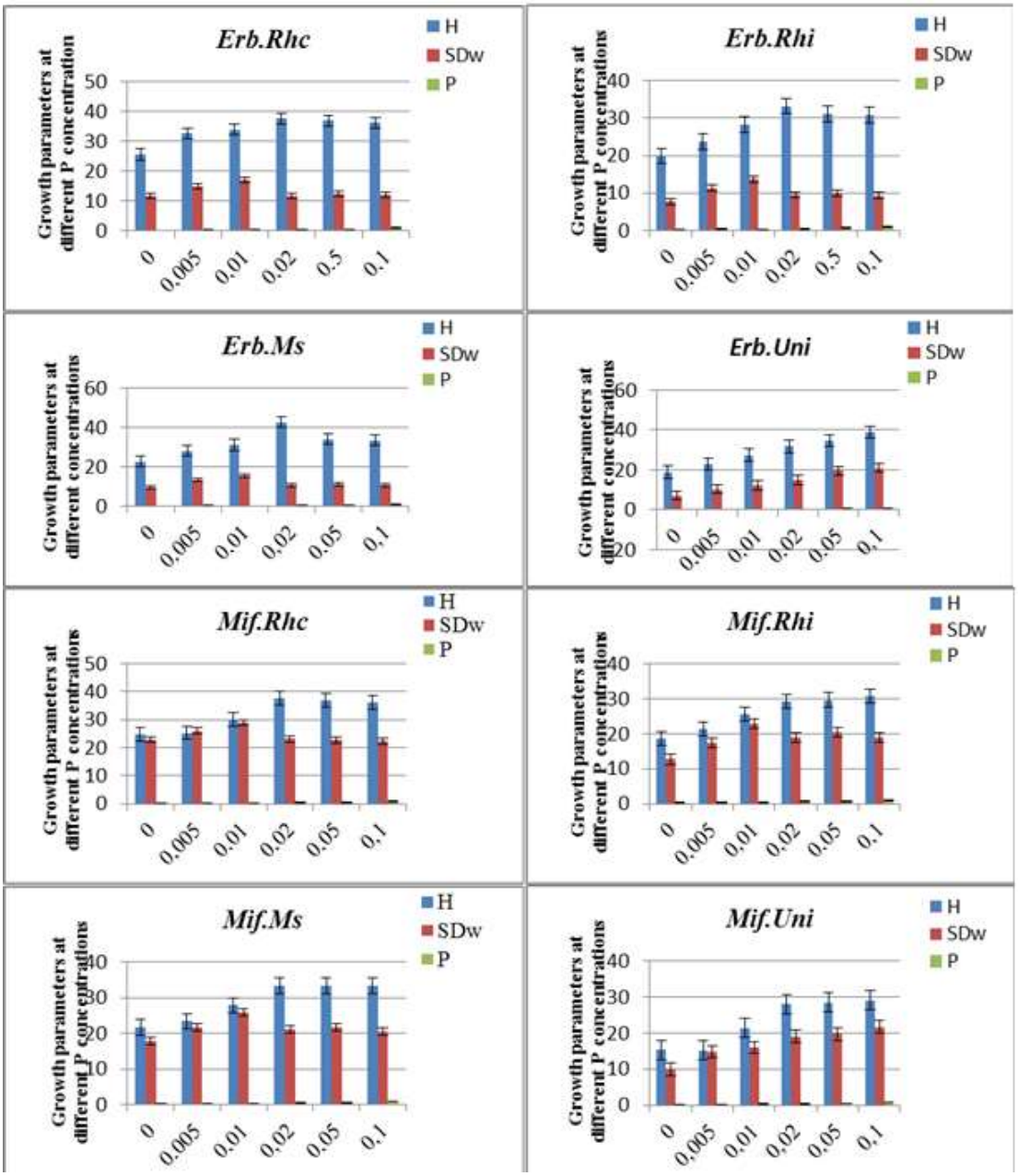

Figure 1. Plant height, shoot dry weight and P uptake at different P concentrations (mg/g) in AMF inoculated and un-inoculated treatments. Key: H, plant height; SDW, shoot dry weight; P, phosphorus; Erb., *Erythrina brucei*; Mif, *Millettia ferruginea;* Rhi., *Rhisophagus intraradices;* Rhc., *Rhizophagus clarus;* Ms., mixed species*; uni.,* un-inoculated.

Table 1. Total shoots dry weight and mycorrhizal dependency (MD) of the trees.

Plant species	MD%						Un-inoculated
	Rh. clarus		*Rh. intraradices*		Mixed species		
	Total SDW	MD	Total SDW	MD	Total SDW	MD	SDW
Erythrina brucei	79.7^{b}	26.19^{a}	61.5^{b}	4.34^{ab}	70.6^{b}	16.67^{b}	58.83^{b}
Millettia ferruginea	146.03^{e}	34.87^{c}	111^{e}	14.32^{e}	128.52^{e}	26.0^{cd}	95.11^{c}

SDW, shoot dry weight; MD, mycorrhizal dependency. Means in the same column followed by different letter(s) are significantly different by ANOVA and Duncan's Multiple Range Test at P<0.05 level.

Table 2. Effects of different phosphorus concentrations (milligrams per gram) on AMF structural colonization (after 1st, 2nd and 3rd months of growth).

Plants	P (mg/g)	*Rhizophagus clsarus*						*Rhizophagus intraradices*						Mixed					
		AC%			VC%			AC%			VC%			AC%			VC%		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Erythrina brucei	0	−	-	+	−	−	+	-	+	+	−	+	+	−	+	+	−	+	+
	0.005	−	+	++	−	+	+	+	+	+	-	+	+	+	+	++	-	+	+
	0.01	+	++	++	−	+	++	+	+	++	-	+	++	+	+	++	-	++	++
	0.02	+	++	++	−	++	+++	+	+	+	-	++	++	+	+	++	-	++	+++
	0.05	−	−	++	+	+	+	−	+	+	−	+	+	−	+	+	−	++	+
	0.1	−	−	+	−	−	+	−	−	+	−	+	+	−	−	+	−	−	+
Millettia ferruginea	0	−	-	+	−	−	+	−	+	+	−	+	+	−	+	+	−	+	+
	0.005	+	++	++	−	+	++	+	+	+	+	+	++	+	+	++	-	++	++
	0.01	+	++	++	+	++	+++	+	+	++	−	+	++	+	+	++	-	++	++
	0.02	+	+++	+++	−	++	++	+	+	+	+	++	+++	+	+	++	-	++	+++
	0.05	−	−	++	−	−	+	−	+	+	−	+	+	−	+	+	−	+	+
	0.1	−	−	+	−	−	+	−	−	+	−	+	+	−	−	+	−	−	+

1, 2, 3, number of months of plant growth; A, arbuscuales; V, vesicles; -, absent, +, fair; ++, Moderate; +++, high.

Table 3. Effects of different phosphorus concentrations (milligrams per gram) on root colonization and spore density (after three months of growth).

Plant species	P mg/g	*Rhizophagus clarus*		*Rhizophagus intraradices*		Mixed species	
		RLC (%)	SD/50 cm^3 soil	RLC (%)	SD/50 cm^3 soil	RLC (%)	SD/50 cm^3 soil
Erythrina brucei	0	12.33^{ab}	25.33^{b}	16.33^{bc}	32.67^{b}	20.00^{bc}	26.17^{b}
	0.005	17.50^{bc}	30.67^{b}	22.67^{cd}	35.67^{b}	23.33^{c}	36.67^{cd}
	0.01	32.67^{d}	57.33^{c}	33.33^{e}	56.67^{c}	33.33^{d}	39.33^{d}
	0.02	21.33^{c}	49.67^{c}	25.67^{de}	42.33^{b}	20.50^{bc}	32.33^{c}
	0.05	10.33^{ab}	6.67^{a}	9.00^{ab}	13.33^{a}	14.67^{b}	9.00^{a}
	0.1	5.17^{a}	3.33^{a}	4.17^{a}	6.67^{a}	3.00^{a}	7.50^{a}
Millettia ferruginea	0	13.33^{b}	26.67^{b}	16.50^{bc}	36.67^{b}	19.33^{bc}	26.67^{ab}
	0.005	17.73^{c}	34.00^{b}	23.33^{c}	40.67^{b}	26.33^{bc}	47.67^{c}
	0.01	28.67^{d}	62.33^{c}	36.33^{d}	69.33^{c}	39.00^{d}	69.33^{d}
	0.02	20.00^{c}	51.00^{c}	24.00^{c}	60.00^{c}	30.33^{cd}	39.67^{bc}
	0.05	11.67^{b}	10.67^{a}	7.67^{ab}	15.67^{a}	17.33^{b}	14.33^{a}
	0.1	6.00^{a}	6.00^{a}	5.33^{a}	10.67^{a}	2.67^{a}	11.00^{a}

RLC, root length colonization; SD, spore density. For each plant species means in the same column followed by different letter(s) are significantly different by ANOVA and Duncan's Multiple Range Test at P<0.05 level.

sand was observed at P concentrations ranging from 0.005 to 0.02 mg g^{-1} in tree seedlings infected by AM fungi (Table 3). In this study, results showed that the P optimum for maximum benefit from AM symbiosis for inoculated agroforestry tree seedlings was in between 0.005 and 0.02 mg g^{-1} and the seedling growth reduced with increasing P concentration.

Therefore, inoculating trees with a suitable AM inoculants could result in a benefit comparable to high P input. However, extrapolation of the results to the real conditions of agroforestry systems should be done with precaution because of differences in the substrate used, that is, sand in the present study. The information on P optimum can form the basis of further pot/field experiments involving integration of chemical fertilizers with AM fungi.

DISCUSSION

Previous studies, in field conditions have shown that

agricultural management practices, such as tillage, fertilization and cropping systems, have a negative impact on the AMF associated with temperate and tropical agronomic plant species (Cardoso and Kuyper, 2006). Fertilization is an important abiotic factor influencing growth, colonization, sporulation, composition and distribution of AMF (Wang et al., 2009).

Other studies conducted in green house conditions (Habte and Manjunath, 1991) have demonstrated that AM fungi usually have their maximum effect on host plant growth when the level of P in the growth medium is optimum. According to Habte and Manjunath (1991), when the soil solution P concentration is at or near 0.002 mg/l, most plant species will respond dramatically to mycorrhizal colonization.

Results of the current study on agroforestry trees and crops revealed the pick for maximum benefit at 0.02 mg P g^{-1} growth medium (sand) and that, as P concentration is increased from 0.005 to 0.02 mg/g, the reliance of plants on AM fungi for P uptake increased and diminished progressively as P concentration increased (from 0.05 to 0.1 mg/g) after which only the very highly mycorrhizal-dependent species respond significantly to mycorrhizal colonization.

Our results also confirm previous results (Ravnskov and Jakobsen, 1995). The mechanism underlying the reduction in plant growth just above optimum P probably includes both effects of P on root growth and direct effects on the fungi (Cardoso et al., 2006). Increase in P supply may decrease the availability of organic substrates from roots to fungi. Azcon et al. (2003) reported that low P concentration in lettuce plants allowed the maximum colonization and occurrence of AM fungi. Koide (1991) showed that P levels influenced AM colonization. Addition of P fertilizers above optimum delayed and/or inhibited AM infection (de Miranda et al*.,* 1989; Baon et al., 1992).

Several other authors have reported that mycorrhizal roots are able to absorb several times more phosphate than non-inoculated roots from soils and from solutions (Nielsen, 1983; Fitter, 1988). Increased efficiency of phosphorus uptake by mycorrhizal plants could have led to higher concentrations of P in the plant tissues. The greater phosphate absorption by AMF has been suggested to have arisen due to superior efficiency of uptake from labile forms of soil phosphate, which is not attributable to a capacity to mobilize phosphate sources unavailable to non mycorrhizal roots (Pearson and Gianinaazzi, 1983). Mycorrhizal roots are known to have not only a considerably greater phosphate inflow rates, but also to possess a pathway of phosphate uptake with a much higher affinity for phosphate than non mycorrhizal roots.

In our study, maximum root colonization and spore count per 50 cm^3 sand was observed at P concentrations ranging from 0.005 to 0.02 mg g^{-1} in plants infected by AM fungi and effectiveness decreased with increasing P concentration. Our results support reports by Kahiluoto et al. (2000) who observed that with increasing P supply, there was a decrease in the colonization and the effectiveness of mycorrhizal colonization. The results are also in agreement with many reports which suggest that addition of phosphate fertilizers above optimum levels results in a delay in infection and reduced chlamydospore production by AM fungi (Koide, 1991; Thingstrup et al., 1998).

In general, the trees studied are fast growing plants, requires more nutrients during the initial stage of seedling establishment. During this period, the root system is not well developed and the AM fungal symbiosis might play a vital role by supplying the nutrients to the host plant (Muthukumar and Udaiyan, 2006). The results of present study showed that mycorrhizal inoculations increased the plant growth and P uptake in different treatments with a few exceptions. This can be due to increase in the sand volume explored for nutrient and water uptake by the mycorrhizal plants from the medium as compared to non-mycorrhizal plants. Our results support previous studies.

The high rate of P fertilizer application, that is, 0.05 and 0.1 mg g^{-1} lead to antagonistic inhibition of mycorrhizal colonization whereas in lower dose with application of the vigorous AM fungi *Rh. intraradices*, was able to increase the root colonization and spore density significantly. However, increased P supply increased some growth parameters connected to plant height, shoot and root dry weight. Thus, soil amendment with AM fungi have the potential to possibly reduce the application of phosphorus fertilizer for crop improvement, growth, yield and nutritional value of the perennial crops and shade trees in Sidama agroforestry.

Our results indicated that inoculating plants with a suitable AM inoculant could result in a benefit comparable to high P input. However, extrapolation of the results to the real conditions of agroforestry systems should be done with precaution because of differences in the substrate used, i.e., sand in the present study. The information on minimum P concentration for better performance of AMF in the agroforestry can form the basis for further pot/field experiments involving integration of chemical fertilizers with AM fungi.

Conclusion

The present study demonstrated that inoculation of multipurpose shade trees with *Rh. intraradices, Rh. clarus* and mixture inoculums of the two, increased all plant growth parameters, but at the same time decreased percentage of mycorrhizal colonization and spore density as the concentration of P increased. Thus, soil amendments with AM fungi have the potential to possibly reduce the application of phosphorus fertilizer for tree and crop growth and improvement in agroforestry. However, in order to come up with more accurate and

reliable information on functional efficiency of the AMF species applied, further pot and field experiment should be carried out.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Survey of Turcicum leaf blight (*Exserohilum turcicum*) on maize (*Zea mays*) in major highland and mid-altitudes of maize growing agro-ecologies of Western part of Oromia, Ethiopia

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Turcicum leaf blight (*Exserohilum turcicum*) (TLB) is a major disease affecting maize (*Zea mays*) in western Ethiopia. This study was designed to: assess the incidence and severity of TLB in major highland and mid-altitudes of maize growing agro-ecologies of Western Oromia, map the geographical distribution of the TLB disease in the study area, and evaluate the reaction of maize varieties under production to TLB. From each representative zone, 2 - 12 districts were surveyed based on production of maize and 8 - 73 fields each averaging 1 ha at the interval of 5 - 10 km were assessed per zone. Such fields were randomly selected on both sides of the road. Each sampling point was marked with the global positioning system (GPS) using GPS receiver for altitude and co-ordinates. In each field, 9 m² areas were marked out in three randomly selected points in a diagonal form using meter tap. Ten stands of maize plant in the middle of each marked area were randomly selected and assessed for incidence and severity. Survey was conducted on 172 farmers' fields in 29 districts and five zones of major maize growing agro ecologies in western Oromia region of Ethiopia. Survey results revealed the prevalence of TLB across all the districts albeit at different levels. Mean TLB incidence ranged from 16.3% in Abay chomen to 96.67% in Wayu Tuka and mean percent severity index varied between 3.1% in Abay chomen and 57.5% in Lalo Assoabi district. The overall mean incidence and PSI were high (up to 74.1 and 34.7%, respectively) during the grain filling stage of maize compared to tasseling and silking stage of the crop. Most of maize varieties under production were found to be affected by TLB, while fertilizer application reduced the intensity of disease. Successive survey for TLB in all the maize producing areas across the country should be carried out to have a complete picture on the importance of the disease across geographic regions and agro ecologies, to identify sources of resistant genotypes and the pathotypes/available in Ethiopia, and to associate weather variables with the development of TLB.

Key words: Distribution, *Exserohilum turcicum*, maize, turcicum leaf blight, Incidence, percent severity index.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most widely grown crops in the world, ranking third next to wheat and rice (Reeves et al., 2016). In sub-Saharan Africa (SSA), maize is the most widely-grown staple food crop

occupying more than 33 million hectares each year (FAOSTAT, 2015). It is among the most important and widely grown crops in Ethiopia, ranking first in total production with over 7.23 million tones of produce and second in area coverage next to Teff (*Eragrostis tef* (Zucc.) Trotter) (Mosisa et al., 2012; Central Statistical Authority (CSA), 2014). Considering its importance, wide adaptation, total production and productivity, maize is regarded as one of the high priority food security crops in Ethiopia, the second-most populous country in SSA after Nigeria (CSA, 2011). However, maize production has remained low, with the estimated national average yield of 3.4 t ha^{-1} (CSA, 2014) compared to the world average yield estimated at 5 t ha^{-1} (FAOSTAT, 2015), due to several major diseases, including foliar diseases.

Turcicum leaf blight (TLB) is a major foliar disease of maize in most production areas worldwide (Jakhar et al., 2017). It is a fungal disease caused by *Exserohilum turcicum* (Pass.) K. J. Leonard and E. G. Suggs. The pathogen was formerly known as *Heliminthosporium turcicum* Pass (Khedekar et al., 2010). The disease is more prevalent in humid areas with moderate temperatures (Pataky et al., 2006). It is widely distributed, however, sporadic in nature and its development mostly depends on weather conditions, stage of plant growth and level of resistance in maize cultivars (Perkins and Pedersen, 1987). The pathogen has wide host range and a high pathogenic variability (Muiru et al., 2010).

The pathogen attacks all parts of the plant but the most conspicuous symptoms/lesions are found on the foliage. Lesions destroy the leaves, resulting in yield losses due to lack of carbohydrate to fill the grains. Heavily infected fields present a scorched or burnt appearance resulting in premature death of leaves (Harlapur et al., 2007). TLB causes extensive leaf damage and defoliation during the grain filling period, and yield losses due to necrosis or chlorosis of leaves premature death of the leaves and loss of nutritive value even as fodder (Patil et al., 2000) has been reported. Yield losses of up to 28 to 91% due to TLB have been reported in Italy, mostly when heavy infection occurred before tasselling (FAO, 2010).

Ramathani et al. (2011) reported the prevalence of TLB in highlands and wetter areas of the Kenya and Uganda. Previous reports have also shown that *E. turcicum* is a serious pathogen in highlands associated with cool, high relative humidity, mid-altitudes and cloudy weather conditions (Palaversic et al., 2012). Extreme impact of TLB on maize in the highland agro-ecologies have also been reported in Uganda, Kenya, Ethiopia and Zambia and South Africa (Ramathani et al., 2011).

In Western Ethiopia, TLB (*E. turcicum*) is the most important maize disease reported. Farmers in this area (46.7%) indicated TLB as the major leaf disease on maize while gray leaf spot *Cercospora zeae-maydis*, GLS, is ranked as the second most important leaf disease in the area (Wende et al., 2013). Therefore, TLB ranked as the number one problem and is considered as a high research priority of maize in Ethiopia (Wende et al., 2013).

TLB incidence ranges from 95 to 100% in areas with constant moisture and high humidity and the yield loss can reach up to 70% (Tewabech et al., 2012). It is reported to cause devastating damage on most commercial varieties of maize released in the country (Tewabech et al., 2012).

TLB varies in incidence and severity from year to year and from one locality to another depending largely on genetic makeup of the plants and prevailing environmental conditions. It is a multiple cycle disease and new repeated inoculations are needed for disease development making it highly dependent upon sporulation from other lesions (Ullstrup, 1966).

Previously, the TLB disease was limited to specific areas and varieties; but currently the disease has become very important almost in all maize growing agro-ecologies. Most released varieties under production are affected by the disease. Although, it becomes very important in the nation, there is limited quantified data that reflect the extent of its distribution across highlands and mid-altitude maize growing agro ecologies and the reaction of maize varieties to this disease under this production system need to be studied. Thus, the present investigation was undertaken with the following objectives:

1) To assess the incidence and severity of TLB in major highland and mid-altitudes of maize growing agro-ecologies of Western part of Oromia, Ethiopia.
2) To map the geographical distribution of the TLB disease in the study area.
3) To assess the reaction of maize varieties under production to TLB in surveyed area.

MATERIALS AND METHODS

Study areas and sampling system

Field surveys were carried out during the 2017 growing season in major maize growing agro-ecologies of Western part of Oromia region of Ethiopia (Figure 1). West Shewa, South West Shewa, Horro Guduru Wollega, East and West Wollega zones were assessed to determine the incidence and severity of TLB of maize. Zones and districts were selected based on the differences in production (farming) systems, weather condition (Relative humidity, maximum and minimum temperature), altitudes and major vegetation cover (availability of maize crop) (Ramathani et al., 2011).

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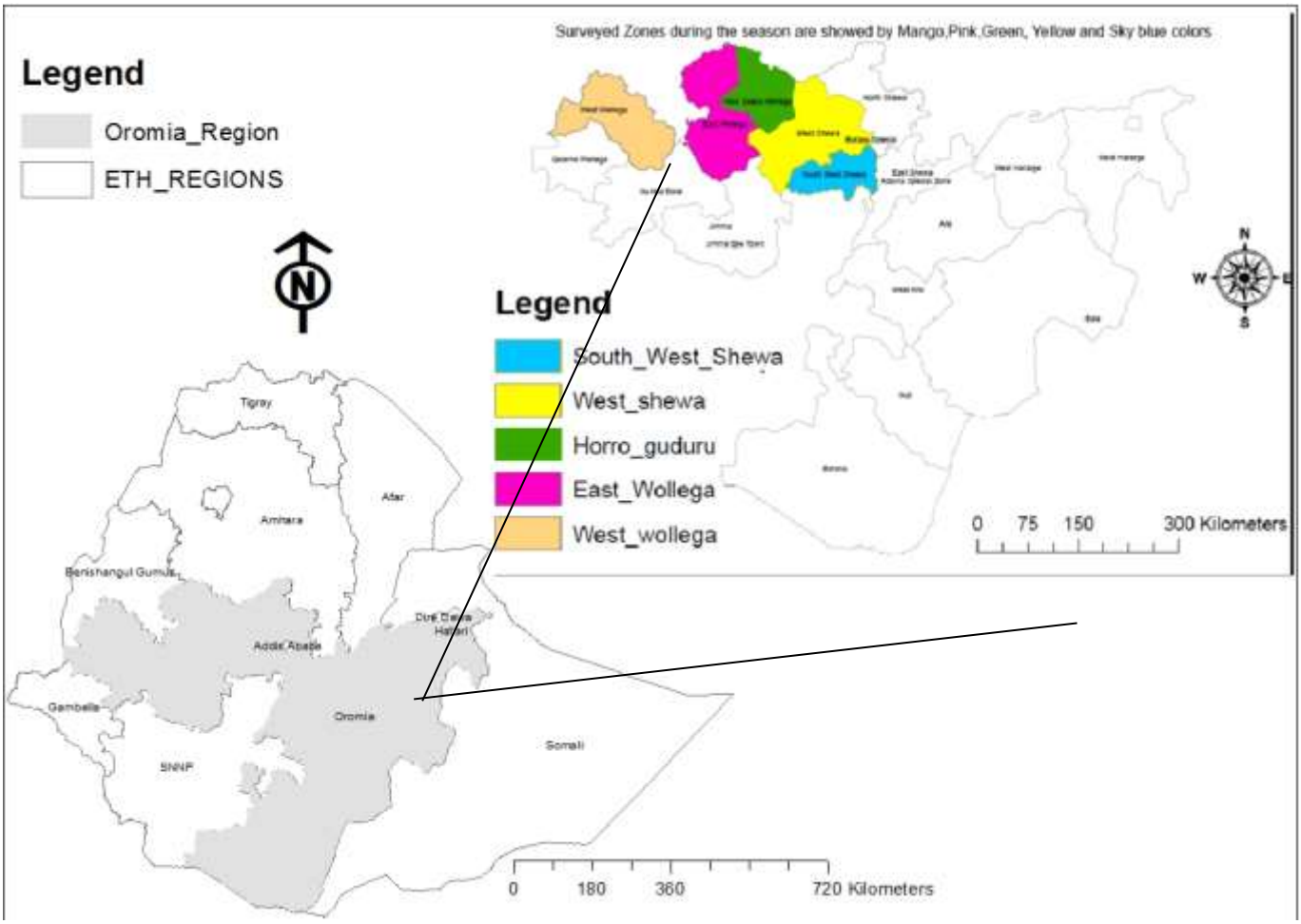

Figure 1. Map of Ethiopia showing the survey area.

However, some farming systems tend to overlap between districts.

From each representative zone, 2 - 12 districts were surveyed based on production of maize crop and 8 - 73 fields each averaging 1 ha at the interval of 5 - 10 km along road side were assessed per zone. Such fields were randomly selected on both sides of the road. A total of 172 farmers' fields, 29 districts and 5 zones were surveyed when the crop was at flowering to grain filling stage. Each sampling point was marked with the global positioning system (GPS) using GPS receiver for altitude and co-ordinates. In each field, 9 m² areas were marked out in three randomly selected points in a diagonal form using meter tap. Ten stands of maize plant in the middle of each marked area were randomly selected and assessed for incidence and severity (Nwanosike et al., 2015). Each marked area was regarded as a replicate for disease assessment.

Assessment of Turcicum leaf blight

Diseases incidence

Disease incidence was assessed as proportion of plants showing symptoms in the field. The number of plants within 10 randomly selected stand showing TLB symptoms were counted and expressed as a percentage of the total number of stands per plot using the following formula (Nwanosike et al., 2015).

$$\mathrm{DI}(\%) = \frac{\text{Number of Diseased plants}}{\text{Total number of plants observed}} \times 100$$

Where, DI is disease incidence.

Disease severity

Disease severity on whole plant basis was rated using a visual scale of 0-5; where, 0 = all leaves free from infection, 1= a few restricted lesions on the lower leaves (≤ 5%), 2= several small and large lesions on many leaves (5.1 - 10 %), 3= numerous small and large lesions on many leaves (10.1 - 25%), 4 = many enlarged and coalesced lesions on many leaves above the cob (25.1 - 50%), 5 = several coalesced lesions, leaf showing wilting, tearing and blotching typical blight symptoms (>50%) (Muiru et al., 2007; Durrishahwar et al., 2008). Severity scores were converted to percent disease index (PDI) (Wheeler, 1969; Kumar et al., 2011).

$$\mathrm{PSI}(\%) = \frac{\text{Sum of numerical rating}}{\text{Total number of plant observed x maximum rating}} \times 100$$

Where: PSI is percent severity index. Varieties with ratings; 0 - 1.4 (<30%) were considered resistant, 1.5 - 2.4 (30 - 50%) =

moderately resistant, 2.5 - 5 (>50%) = susceptible (Muriithi, 2001).

Data analysis

Disease data (incidence, severity and PSI) were entered into Excel to calculate means and percentages. Correlation analysis was carried out between disease data and weather parameters using the Proc. Corr. procedures of the SAS software (SAS, 2002). Disease distribution maps were generated using the Arc GIS 10.3 software with spatial analyst by interpolating the surface from GPS points and the associated field severity data using the inverse distance weighted (IDW) interpolation method.

RESULTS AND DISCUSSION

Status of Turcicum leaf blight of maize (TLB) in surveyed areas

Turcicum leaf blight of maize was prevalent in all surveyed agro-ecologies in 2017 cropping season at varying levels (Table 1). The mean TLB incidence in the surveyed districts ranged between 16.3% (Abay chomen district of Horro Guduru Wollega Zone) and 96.7% (Wayu Tuka district of East Wollega Zone). Amongst all the surveyed districts, the highest disease incidence (96.7%) was recorded in Wayu Tuka followed by Lalo Assabi, Sibu Sire, Diga and Bako tibe with disease incidence of 94, 92.5, 91.8 and 90%, respectively. On the other hand, Abay chomen, Horro, Bacho and Jima rare districts had relatively lower TLB incidence of 16.25, 20, 25 and 26.7%, respectively.

Percent severity index (PSI) ranged from 3.13% in Abay chomen district to 57.50% in Lalo Assabi district. The highest mean PSI (57.5%) was recorded in Lalo Assabi district followed by Sibu sire, Bako Tibe, and Toke Kutaye, which had 50.7, 46 and 45% PSI, respectively. In contrast, the mean minimum PSI was recorded in the Abay Chomen district (3.13%) followed by Bacho (4%), Dandi (6.33%) and Jima rare (6.67%).

The highest maize TLB incidence and PSI were recorded in areas with a mean maximum temperature of 31.12 - 34.93°C and humid areas (Table 1). The finding of the present study is in agreement with Nwanosike et al. (2015) who stated that Turcicum leaf blight of maize is widely distributed, and the level of intensity varied across locations in Tanzania and the highest TLB incidence and percent severity index recorded at maximum temperature up to (31 - 35°C) and high relative humidity (75 - 100%). Earlier researchers (Harlapur, 2005; Khedekar et al*.,* 2010; Rani, 2015) suggested that prevailing environmental conditions (relative humidity, maximum and minimum temperature) during cropping season could be reasons for higher disease incidence. Ullstrup (1966) also reported that TLB incidence varies in prevalence and severity from year to year and from one locality to another, depending largely on environmental conditions (that is, humid weather along with heavy dew favored the spread and development of the disease in an epidemic form). TLB has particularly been noticed to cause significant maize yield reduction in many production regions including in sub-Saharan Africa (SSA), especially in the humid mid-attitude and highland regions (Latterell and Rossi, 1983; De Vries and Toenniessen, 2001).

Beshir et al. (2015) reported that *E. turcuicum* was the causative agent of leaf blight observed in central Sudan. Furthermore, the TLB occurred in all the study locations with incidence and severity ranging from 45 to 100, and 65 to 100, respectively.

In Africa, where maize and sorghum are the staple foods, TLB is reported to be widespread in the warm and humid growing regions of Ethiopia, Tanzania and Uganda (Adipala et al., 1993a; Tewabech et al., 2001).

Geographical distribution of TLB of maize in surveyed areas during the season

Disease incidence, PSI and GPS records were used to construct disease map in surveyed zones and districts.The disease map illustrates severity and incidence levels over the agro-ecologies and was used to study epidemic patterns at the time of the study. Results showed spatial pattern of epidemics.

TLB incidence was categorized on the map as described by Harlapur (2005), where 0 - 5% = slight/trace infection, 5.1 - 15% = light infection, 15.1 - 30% = moderate infection, 30.1 - 75 = severe infection, >75 = very severe infection.

In terms of incidence, virtually all districts had very high level of disease. The TLB incidence in vast majority of districts studied recorded disease incidence of 75% and above (Figure 2). In Ambo Zuria, Ilu Galan, Dire Inchini, Nono, Dandi, Dawo, Waliso, Guduru, Kiramu, Gobu Sayo, Guto Gida, and Gimbi districts severe infection (30.1 - 75%) of TLB were recorded which was indicated by orange colour on the map. Toke Kutaye, Sasiga, Diga, Wayu Tuka, Sibu Sire, Bako Tibe, Jibat, Lalo Assabi, Dano, and Chelia recorded above 75% which were very severe infection indicated by red colour on the map (Figure 2). On the other hand, moderate infections (15.1 - 30%) of TLB were recorded in a few districts such as Becho, Abay Chomen, Horro and Jimma Rare. This was due to moderately resistant to resistant hybrid varieties or cultivar grown in the districts.

The disease map also illustrates spatial patterns of epidemics with some districts showing high variation in PSI. PSI levels ranged from as low as 3.13% in Abay Chomen to as high as 57.7% in Lalo Assabi district. It was described on the map according to Nwanosike et al. (2015), where, 0 – 29 = low severity, 30 – 49 = moderate severity and 50 – 100 = high severity (Figure 3).

In general, low value of disease incidence and severity suggests that a number of varieties grown by farmers appear to be resistant or tolerance to TLB. Second

Table 1. Mean incidences and PSI of Turcicum leaf blight on maize across surveyed districts and zones during the 2017 growing season in Ethiopia.

Zone	District	Field No.	Altitude range (m.a.s.l)	Min.T (°C)	Max.T (°C)	RH%	Disease incidence (%)			PSI (%)		
							Mean	Range	St dev.	Mean	Range	St dev.
West Shewa	Bako Tibe	10	1623-1708	22.14	33.90	68.10	91.00	50-100	16.63	46.00	10-75	23.66
	Ilu Galan	8	1707-1859	24.53	31.05	61.38	63.75	30-100	27.22	19.00	10-52	14.96
	Dano	7	1600-1739	24.00	35.59	70.00	75.71	50-100	18.13	31.71	8-60	19.70
	Cheliya	2	2140-2561	23.40	35.60	65.00	80.00	60-100	28.28	32.50	20-45	17.68
	Liban Jawi	4	2269-2455	23.45	35.68	65.00	62.50	30-90	27.54	23.75	10-40	16.01
	Toke Kutaye	11	1947-2416	21.15	33.29	68.82	79.09	30-100	27.00	45.00	10-70	21.56
	Ambo zuria	10	2053-2624	20.45	27.14	67.40	73.00	30-100	26.69	34.10	4-60	21.52
	Dire Inchini	5	2413-2491	16.20	34.72	79.00	72.00	30-100	30.33	32.40	10-55	21.82
	Shenen	1	2495	16.20	36.00	79.00	80.00	80	-	40.00	40	-
	Jibat	5	2100-2565	16.20	36.00	81.00	90.00	60-100	17.32	35.00	10-55	20.31
	Nono	7	1525-2062	19.03	36.00	81.00	68.57	50-100	21.93	22.57	8-60	19.36
	Dandi	3	2257-2592	15.40	36.00	81.00	43.33	30-50	11.55	6.33	4-10	3.22
South West Shewa	Waliso	3	2174-2342	15.40	36.00	81.00	73.33	50-90	20.82	18.33	10-25	7.64
	Bacho	2	2159-2268	15.40	36.00	81.00	25.00	20-30	7.07	4.00	4	0
	Dawo	3	2175-2203	15.40	36.00	81.00	43.33	40-50	5.77	8.66	8-10	1.16
East Wollega	Diga	11	1193-2237	27.89	31.63	57.00	91.82	80-100	7.51	39.27	25-60	14.62
	Kiramu	4	1987-2084	22.20	26.25	50.50	70.00	0-100	46.90	42.50	0-65	29.58
	Gida Ayana	15	1267-1911	23.04	28.49	52.67	80.00	40-100	22.04	38.93	10-75	26.08
	Guto Gida	3	1342-1394	28.40	33.77	57.00	53.33	30-100	40.41	30.00	10-55	22.91
	Wayu Tuka	3	1888-2067	21.87	34.93	67.00	96.67	90-100	5.77	18.33	10-25	7.64
	Sibu sire	8	1722-1834	22.40	36.30	78.38	92.50	80-100	8.86	50.75	10-70	17.56
	Gobu Sayo	3	1746-1857	19.43	31.60	59.67	66.67	20-100	41.63	42.33	5-70	33.56
Horro Guduru Wollega	Horro	3	2390-2400	29.80	31.70	61.33	20.00	0-30	17.32	6.67	0-10	5.77
	Abay choman	8	2308-2418	28.74	31.70	67.25	16.25	0-40	15.05	3.13	0-5	2.59
	Guduru	4	2251-2285	25.25	25.43	58.75	35.00	0-80	33.17	10.00	0-25	10.80
	Choman Guduru	2	2229-2302	23.00	28.45	75.00	35.00	30-40	7.07	7.50	5-10	3.54
	Jima rare	6	2267-2622	23.00	31.03	84.00	26.67	20-40	8.17	6.67	5-10	2.58
West Wollega	Gimbi	11	1746-1857	27.04	32.78	64.73	67.27	40-100	21.95	32.27	10-60	16.64
	Lalo Assabi	10	1773-1900	25.67	31.12	75.10	94.00	60-100	13.49	57.50	25-75	15.68

PSI=percent disease index, RH=relative humidity, Min.T=minimum temperature, Max.T=maximum temperature,St dev. = Standard deviation.

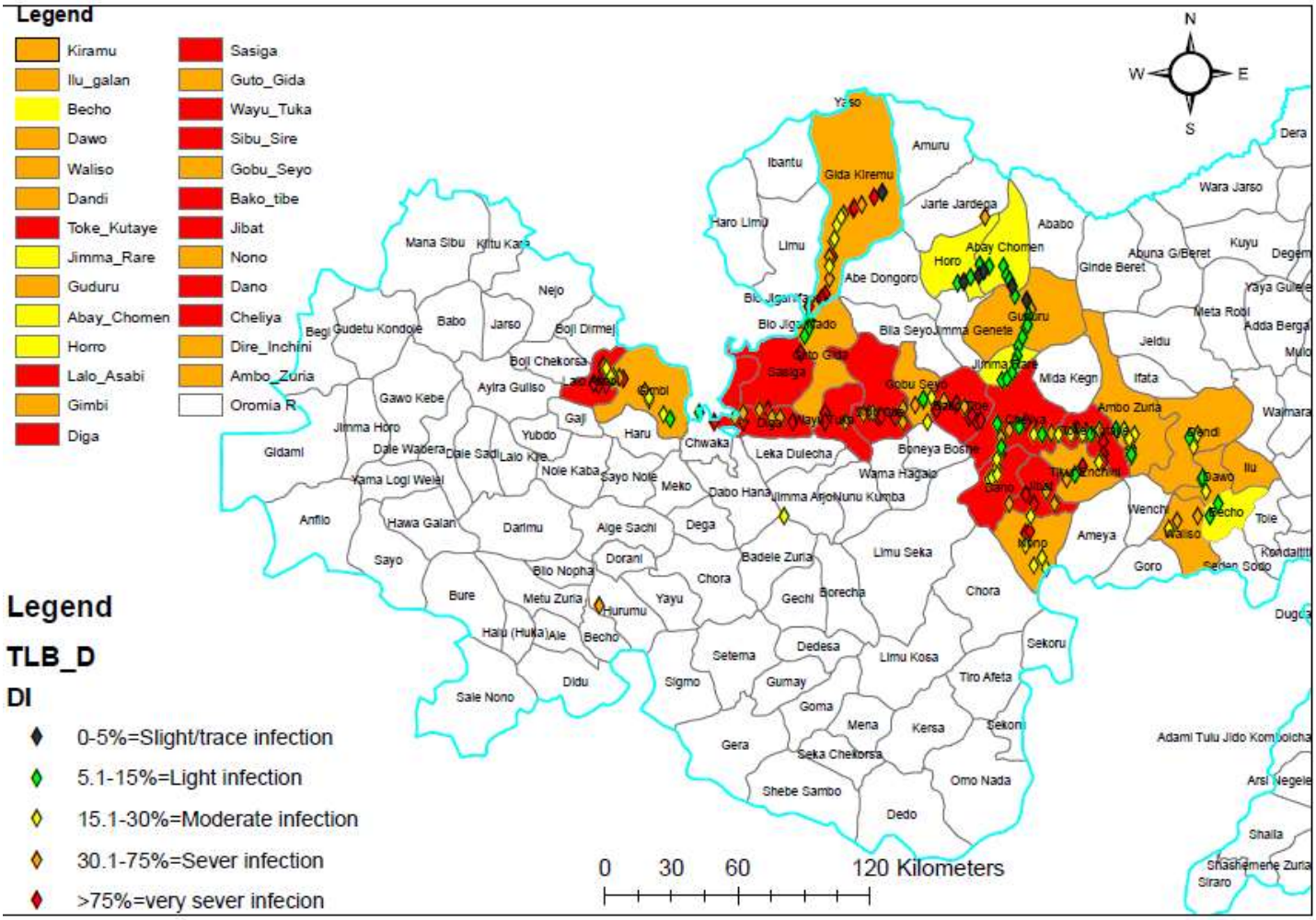

Figure 2. Disease map based on incidence of Turcicum leaf blight of maize in 29 districts of (5 different Agro-ecologies of zones) of Western Oromia region of Ethiopia during 2017 main growing season.

possible explanation for the observed data could be crop rotation (especially preceding crop).

The distribution of TLB and pathotypes of *E. turcicum* have been identified in Kenya (Ngugi et al., 2000) and Uganda (Sserumaga et al., 2013). According to Wende et al. (2013), Turcicum leaf blight is ranked as the number one problem and is considered a high research priority of maize in Ethiopia.

Status of TLB in relation to maize varieties under production

Survey result on incidence and percent severity index of TLB revealed that maize varieties under production varied in their reaction to TLB. The highest mean disease incidence (90%) was scored on variety designated as Oromia, whereas the lowest (30%) was recorded on variety denominated as Shashamane (Figure 4). Varieties such as Limu, Wenchi and BH-540 had 84, 80 and 79.1% disease incidences, respectively. TLB incidences of 37.5 and 47.5% were recorded on unspecified hybrid and Jibat varieties, respectively.

TLB index expressed as PSI ranged from 10% on variety Shashamane to 42% on BH-540. Maize varieties Limu and Kolba also had higher PSI of around 40%. The study revealed that TLB was more common and severe on varieties such as BH-540, Limu, Kolba and Local. In contrast, varieties such as Shashamane, Global and Jibat performed relatively better than others with respect to the TLB reaction in the surveyed areas (Figure 4). This indicated that the upsurge in the TLB on maize was highly influenced by the nature of susceptibility/resistance of the varieties and conducive environmental conditions for the development of the disease in the location. Earlier survey reports (Harlapur et al*.,* 2000; Ramathani, 2011) indicated that cultivar susceptibility and weather parameters play an important role in influencing TLB

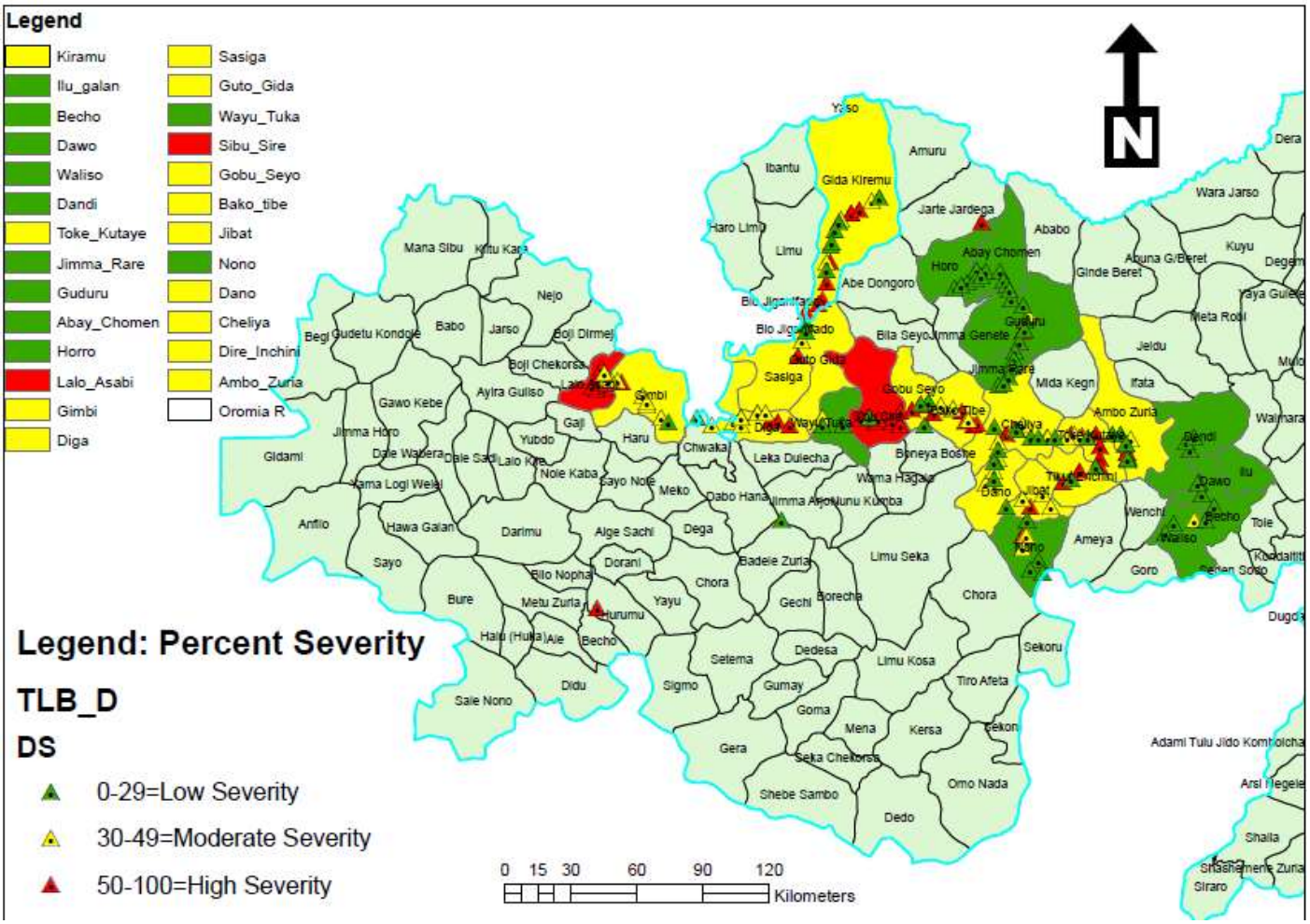

Figure 3. Disease map based on percent disease index of Turcicum leaf blight of maize in 29 districts (5 different Agro-ecologies of zones) of Western Oromia region of Ethiopia during 2017 main growing season.

severity. Turcicum leaf blight is reported to cause devastating damage on most commercial varieties of maize released in Ethiopia (Tewabech et al., 2012).

Krausz et al. (1993) reported an epidemic of turcicum leaf blight from Texas and reported loss in grain yield of susceptible hybrids ranging from 40 to 50%, whereas hybrids with either Ht1 resistance gene or significant polygenic resistance had minimum disease incidence. Babu et al. (2004) reported TLB incidence on maize at Almora and it attained epidemic proportion resulting in 83% yield reduction.

Intensity of TLB at different growth stages of maize

TLB incidence and PSI showed marked variations across the different maize growth stages. This revealed that the development of TLB also relies on the maize growth stage. The overall mean incidence and PSI were high (up to 74.1 and 34.7%, respectively) during the grain filling stage of maize compared to tasseling and silking stage of the crop (Figure 5). Kalappanavar (2017) conducted survey in India and reported that the maximum disease severity was recorded at grain filling stage (22.06 and 15.50%) and minimum at vegetative stages (14.31 and 9.49%).

From the current study, data higher yield loss due to TLB could be expected in many of the surveyed location because the disease occurred before silking stage of maize. This is in agreement with the study of Raymond and Hooker (1981) who stated that if the TLB disease establishes before silking, yield reduction of up to 40% may occur but if infection delays until 6 - 8 weeks after silking, yield losses would be minimal. The present study confirmed wider distribution of TLB in surveyed areas as associated with plant stages. This is in line with Perkins et al. (1987) who stated that TLB disease is widely distributed; however, sporadic in nature and mostly depends on weather conditions, stage of plant growth and level of resistance in maize cultivars.

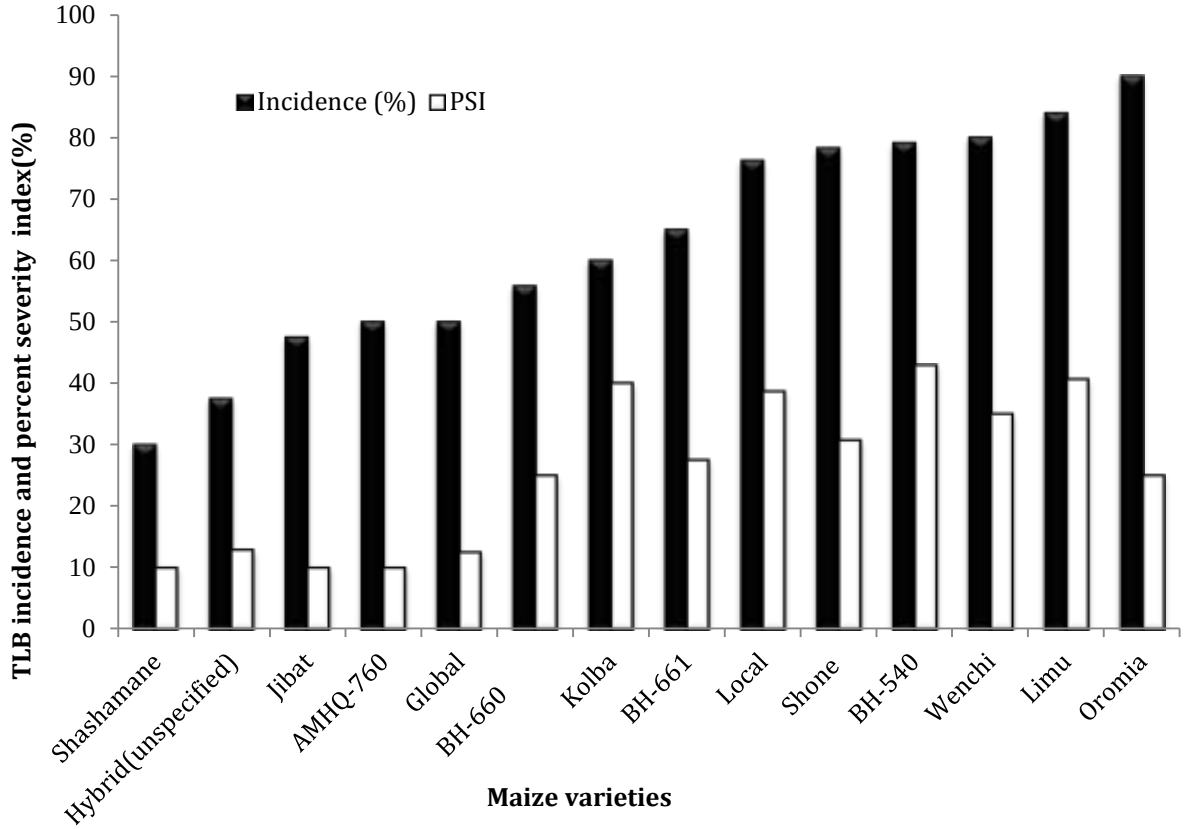

Figure 4. Incidences and percent severity index of Turcicum leaf blight on maize varieties under production in surveyed districts during 2017 main growing season in Ethiopia.

In Uganda, overlapping of growing seasons and presence of off-season maize resulted in infection before tasseling stage, consequently resulting to higher yield loss (Adipala et al., 1993b). Yield loss is caused predominantly through loss of photosynthetic leaf area due to blighting (De Vries and Toenniessen, 2001). Loss of photosynthetic tissue can result in decreased yield, and silage quality can be affected (Jakhar et al., 2017).

Effects of cropping system and preceding crop on maize TLB incidence and PSI

Types of cropping system, previous crop, and residue left in the field played important role for the development of maize TLB in the surveyed areas. PSI ranged from 12.3 to 60% during the current survey (Table 2). The maximum PSI was recorded in fields where maize is intercropped with sorghum while maize-kale intercropping was associated with the lowest PSI. On the other hand, most of the intercrops failed to reduce TLB level as compared to mono cropping. The maximum TLB severity in sorghum-maize intercropping might be due to sorghum's ability to serve as collateral host for *E. turcicum,* the cause of TLB. However, no definite pattern was established in the current experiment as far as the effect of intercropping on TLB is concerned. Assefa (1997) also reported that planting maize and sorghum together showed higher TLB intensity and lower common rust (CR). Ramathani et al., (2011) also suggested that cropping systems significantly influenced the patterns of spread of TLB disease.

With respect to effect of preceding crop, the current work failed to establish a pattern with respect to effect of rotation on TLB development. The only exceptions were maize following fenugreek, which had 30% incidence and 5% PSI, a much lower score as compared to other systems. TLB is residue born disease; as a result, alternatively sowing non cereal crops in the same field can reduce the availability of host residue from the field, thereby affecting the chance of the pathogen to over season. This obviously reduces the inoculum level (source) and the incidence of early season development of the disease. Studies on TLB on maize indicate that maize residues are an important factor for the survival of the pathogen and initiation of the epidemic (Fullerton and Fletcher, 1974). Assefa (1997) stated that maize planting after maize would result in high TLB intensity, while planting maize following noug-maize-noug (Niger Seed) system result in lower severities of TLB. High percent

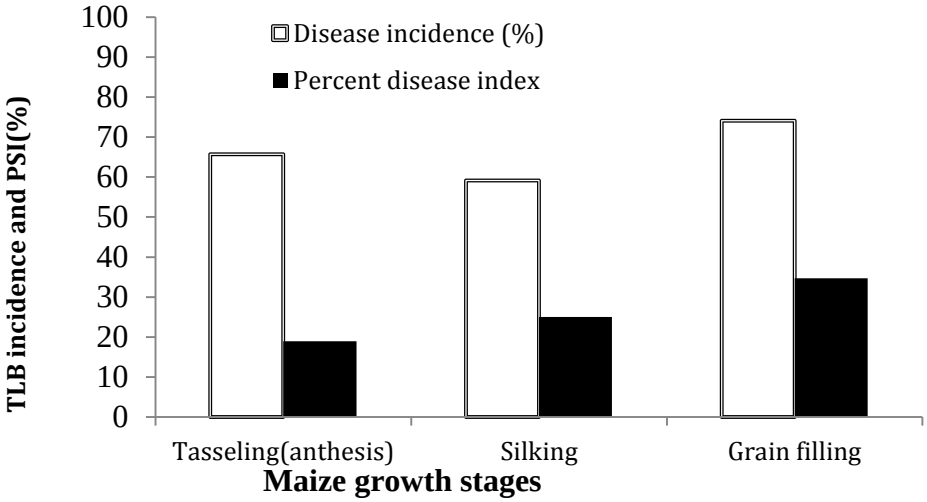

Figure 5. Mean incidence and percent severity index of Turcicum leaf blight on maize at different growth stages of maize growing in surveyed areas during 2017 main growing season in Ethiopia.

severity index on maize was due to intensive cultivation of maize crop season after season, every year, narrow genetic makeup of the commercial hybrids and non-adoption of disease management practices by the farmers (Kalappanavar, 2017). Increased severity of Turcicum leaf blight in Africa associated with continuous cultivation of maize and susceptible maize cultivar has also been reported by Danson et al. (2008). However, lack of marked reductions in TLB level across most of the rotation schemes, included in the current survey, might be because of short durations for rotating maize with other crops. The fact that most of the rotating crops were cereals and might have also contributed to the lack of any significant effect by the rotation scheme on TLB development.

TLB incidence and percent disease index on maize in relation to fertilizer application

Fertilized and non-fertilized maize fields assessed during the survey varied in the level of TLB incidence and PSI. The TLB disease incidence and PSI were relatively low in fertilized fields compared to non-fertilized fields (Figure 6). Mean minimum TLB incidence (67.7%) and PSI (29.9%) were recorded from fertilized fields. On the other hand, maximum disease intensity, 86.7% TLB incidence and 44.8% PSI were recorded in non-fertilized maize fields (Figure 6). It is known that plants suffering from nutrient deficiencies are weaker, slow in growing, and faster in aging. Such plants are susceptible to pathogens (Agrios, 2005). Assefa (1998) studied the effects of farm-yard manure (FYM), and nitrogen and phosphorus rates (N/P) on the intensity and frequency of Turcicum leaf blight and reported that the incidence was low (26.4%) at 20/46 kg/ha N/P_2O_5 plus 24 t/ha FYM as compared to the other combinations (with a maximum incidence of 33.6%) and the two checks (40/46 and 75/75 kg/ha N/P_2O_5). Plant population and fertilize application influence the incidence and severity of Turcicum leaf blight of maize. TLB of maize percent disease index was significantly reduced in treatments that received optimum fertilizer dosage compared to others (Kumar et al., 2017).

Maize TLB incidence and percent severity index in relation to planting date

Disease incidence and percent severity index were low in maize fields planted late April. The minimum disease incidence (50%) and percent severity index (7%) were recorded in maize fields planted in this time followed by mid-May where TLB incidence and percent severity index were 51.5 and 18.25%, respectively. The maximum disease incidence and percent severity index of TLB were observed on maize fields sown at early April and mid-June. At early April and mid-June the percent severity index recorded was 55%. But, disease incidence showed slight difference 100 and 80%, respectively (Figure 7). This very high and low severity of TLB disease is due to the environmental conditions that favour the development of disease and increase the incidence and severity on the maize during this growing season. This study indicated that early and late planting date favoured TLB development (both TLB incidence and severity high in maize). Knowing proper planting date can help the farmers to escape the maize crop from the time when the epidemics of this disease outbreaks. Timely planting can often help cultivars escape the most severe damage from TLB if crop development outpaces normal disease

Table 2. Mean incidences and percent severity index of Turcicum leaf blight on maize under different production systems in major maize growing areas during 2017 main growing season in Ethiopia.

Particulars	No. of fields	DI (%)	PSI (%)
Cropping system			
Mono cropping	50	69.936	31.854
Inter cropping			
Maize (*Zea mays*) with Ethiopian Kale (*Brassica carinata*)	25	43.33	17.33
Maize with ground nut(*Arachis hypogaea*)	19	100	55
Maize with haricot bean(*Phaseolus vulgaris)*	24	96.67	31.67
Maize with Faba bean(*Vicia fabae*)	15	53.33	12.33
Maize with sorghum (*Sorghum bicolor*)	25	90.00	60.00
Maize with potato (*Solanum tuberosum*)	14	100.00	55.00
Previous crop (preceding crop)			
Barley (*Hordeum vulgare*)	6	80.00	35.40
Dagusa, Finger millet (*Eleusine coracana)*	20	100.00	40.00
Ethiopian kale, Gomen, (*Brassica carinata*)	10	60.00	20.00
Faba bean(*Vicia faba*)	8	61.00	21.20
Fallowing	9	62.5	15.75
Fenugreek (*Trigonella foenum-graecum*)	20	30.00	5.00
Groundnut(*Arachis hypogaea*)	9	100.00	55.00
Haricot bean(*Phaseolus vulgaris*)	12	70.00	27.00
Maize(*Zea mays*)	17	72.61	34.28
Noug (*Guizotia abyssinica*)	5	55.63	24.19
Pea (*Pisum sativum)*	4	80.00	25.00
Pepper (*Capsicum frutescens*)	5	82.00	47.00
Potato (*Solanum tuberosum*)	6	50.00	21.00
Rice (*Oryza sativa*)	8	100.00	25.00
Sesame (*Sesamum orientale*)	10	100.00	70.00
Sorghum (*Sorghum bicolor* L.)	15	82.50	38.75
Teff (*Eragrostis tef*)	4	71.33	31.40
Wheat (*Triticum aestivum*)	4	60.00	23.00

DI=Disease incidence, PSI= percent severity index.

progression. The latest-planted maize in an area may be infected when plants are smaller, resulting in the disease progressing more rapidly relative to the crop. However, in cases of high disease incidence, both early- and late-planted maize may be severely damaged by TLB (Jakhar et al., 2017). Assefa (1997) reported that planting maize before May 18 could result in a lower incidence of TLB.

Correlation between Turcicum leaf blight of maize and weather parameters

TLB incidence and PSI positively correlated with relative humidity although not significantly differ (Table 3). On the other hand, TLB incidence negatively correlated with maximum and minimum temperatures. Similarly TLB PSI was negatively correlated with maximum and minimum temperatures, while altitude had significant and negative relationship with both TLB incidence and PSI. In general, the disease levels were significantly influenced by altitude but not weather conditions according to results of the current survey. Reddy et al. (2013) observed negative correlation between disease incidence and maximum temperature and positive correlation with high relative humidity. They reported that the mean minimum temperature had no effect on the disease development.

The results are contrary to those reported by Nwanosike (2015) in which TLB was highly influenced by relative humidity. Rani (2015) also reported positive and significant relationship between relative humidity and disease intensity and significantly negative correlations between minimum and maximum temperature on one hand, and disease intensity on the other. Rai et al. (2002) have also revealed definite relations between

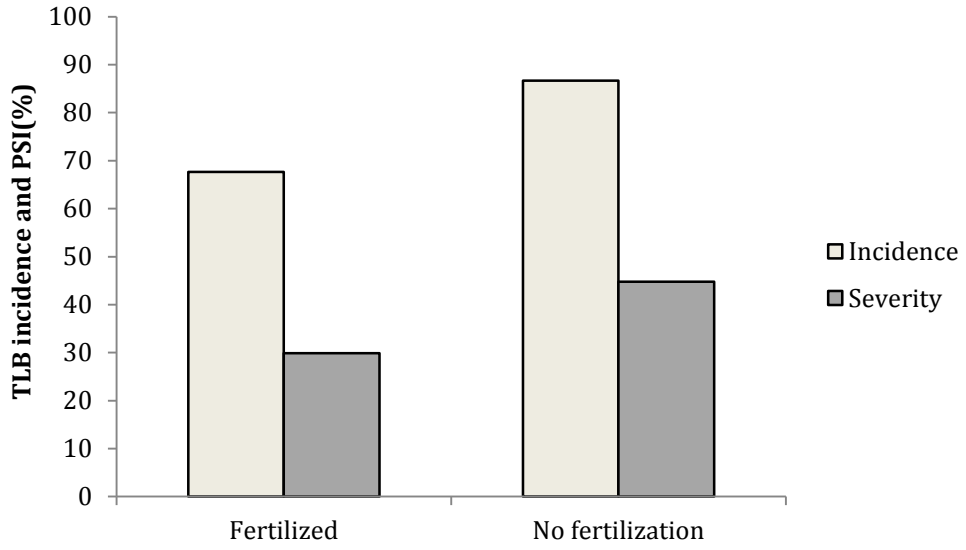

Figure 6. Mean disease incidence and percent severity index of TLB on maize fields with and without fertilizer applications in surveyed areas during 2017 main growing season in Ethiopia.

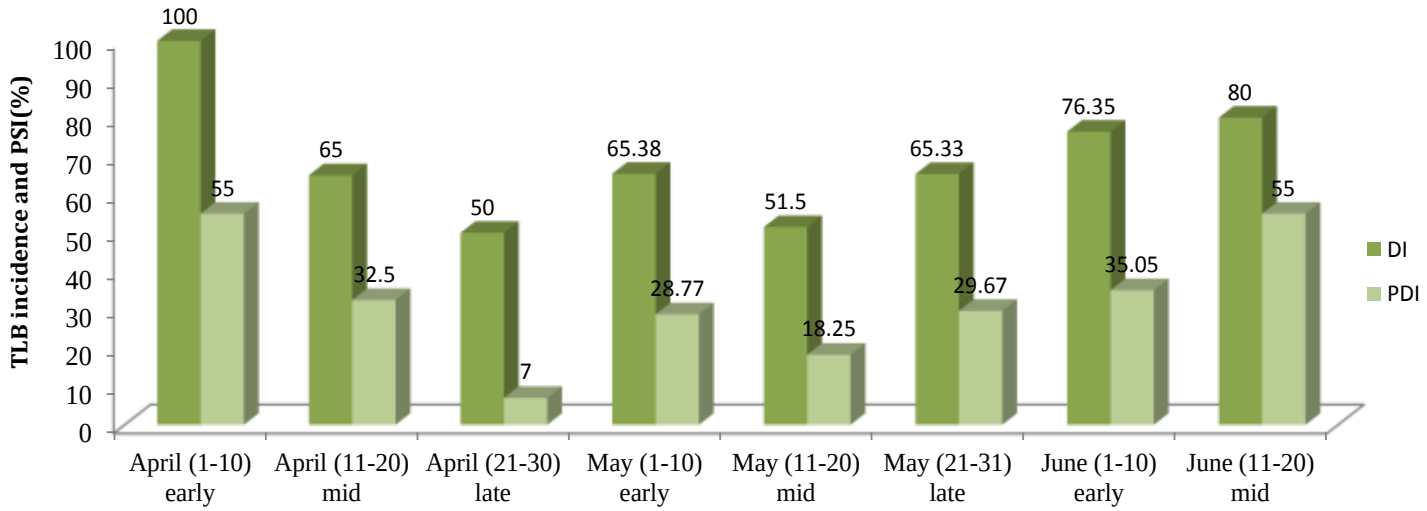

Figure 7. Mean Incidences and percent severity index of TLB on maize at different planting date observed during 2017 growing season in major maize growing surveyed areas.

environmental parameters and disease development with the maximum initial disease incidence (100%) and significant disease severity (4.00) recorded during February.

The deviations of current survey results from those of previous studies might be because weather data were recorded only using portable devices during disease assessment dates in the current survey. It should be noted that disease development in general is influenced by long term and short term weather conditions that span longer than the few particular dates. However, absence of weather stations in the survey areas, make it impossible to have weather records throughout the growing period and beyond. Other reason for this deviation of the area covered in this investigation were mid and highland altitude parts of major maize growing areas in western parts of Oromia regional state only (that is, the lowlands major maize growing areas were not covered in this study).

CONCLUSION AND RECOMMENDATIONS

Disease surveys were conducted in 172 farmers' field, 29

Table 3. Correlation coefficient between TLB disease of maize and weather parameters during assessment in 2017 main growing season in surveyed area.

Variable	Incidence	PSI	Altitude	Max.temp.	Min.temp.	RH
Incidence						
PSI	0.809**					
Altitude	-0.319**	-0.233**				
Max.temp.	-0.069^{NS}	-0.091^{NS}	-0.055^{NS}			
Min.temp.	-0.109^{NS}	-0.064^{NS}	-0.390**	0.025^{NS}		
RH	0.084^{NS}	0.089^{NS}	0.284**	0.068^{NS}	-0.496**	

**Significant at p ≤ 0.01, Maximum and minimum temperature (°C), Relative humidity (%), PDI= percent disease index, Incidence (%).

Assabi districts, respectively. The present study demonstrated that the TLB disease was prevalent and intense across surveyed areas. Most of maize varieties under production were found to be affected by TLB, while fertilizer application reduced the intensity of disease. In addition, late and early planting dates favor TLB epidemic districts and 5 zones in western Oromia region. This study revealed the prevalence of TLB on maize across all the districts although at different levels. Across the survey districts, TLB incidence ranged from 16.25% in Abay Chomen to 96.67% in Wayu Tuka districts. PSI also ranged from 3.13 to 57.50% in Abay Chomen and Lalo development. However, the current work did not demonstrate a clear pattern in terms of effect of intercropping and crop rotation on TLB development except for kale-maize intercropping and fenugreek-maize rotations that resulted in marked reduction in the disease level as compared to other systems. The current investigation revealed the importance of TLB across the study areas. Nonetheless, the following recommendations were made to further enhance the knowledge on the pathosystem:

1) Successive survey for TLB in all the maize producing areas across the country should be carried out;
2) To have a complete picture on the importance of the disease across geographic regions and agro ecologies;
3) To identify sources of resistant genotypes and the pathotypes/available in Ethiopia; and
4) To associate weather variables with the development of TLB.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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