Pre-breeding of Tef [*Eragrostis tef* (Zucc.) Trotter] for Tolerance to Aluminium Toxicity

By

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Thesis Summary

Tef [*Eragrostis tef* (Zucc.) Trotter] is the most widely grown cereal crop in Ethiopia. Its grain is used for human consumption and the straw is an important and highly valued livestock feed. Soil acidity and Al toxicity are among the major production constraints affecting tef in Ethiopia. Utilization of lime and other non-genetic acid soil management options is constrained by various socio-economic factors. Development of cultivars with tolerance to Al-toxicity is a complementary approach to liming in the production of globally important crops such as wheat, rice, maize, barley, sorghum and rye. However, no breeding for tolerance to Al toxicity in tef had been undertaken previously. Hence, this research project was initiated in order to address the following objectives:

1. To assess the perceptions, challenges and coping mechanisms of farmers dealing with soil acidity and Al-toxicity in problem areas of north western Ethiopia;
2. To characterize the reactions of released tef varieties to soil acidity and the associated Al-toxicity;
3. To determine the extent of genetic diversity among tef germplasm collected from areas of Ethiopia with acid soil;
4. To isolate and characterize EMS-induced mutants of tef for tolerance to Al-toxicity and other important agronomic traits;
5. To evaluate the use of hydroponics system as a phenotyping platform to screen for Al-tolerance in tef, using root measurement and haematoxylin assay methods.

There is no information on breeding for Al-tolerance in tef. Therefore, relational background literature was collated on other cereals on their mechanisms of Al-toxicity, tolerance mechanisms, genetic control, screening methods and marker assisted breeding. The information obtained from such sources was used to develop and undertake the subsequent breeding activities on tef.

In order to meet the set objectives, several laboratory, greenhouse, and field experiments were conducted at the Amhara Regional Agricultural Research Institute (ARARI), Ethiopia, from December 2012 to June 2015.

A Participatory Rural Appraisal (PRA) study was conducted in three Districts of north western Ethiopia that are affected by acid soils, in order to assess the state of soil acidity, and to determine its perceived causes and indicators, and to document the coping strategies of the farmers. Semi-structured interviews, group discussions and

soil analyses were the main techniques used to generate data in this background study. Farmers' perceived the causes of soil acidity to include: soil erosion; poor nutrient recycling; the abandoning of traditional fertility management practices; the unbalanced and/or minimal use of external inputs; and the exclusive use of acid-forming, inorganic fertilizers. Soil erosion, soil acidity, the high cost of mineral fertilizers and lime, cash shortages, and a lack of acid tolerant crop varieties were ranked as the top constraints. Species tolerance to soil acidity was found to be one of the major factors that influenced crop choice by farmers. A decline in genetic diversity and the rapid expansions of newly introduced, acid tolerant crops such as oat and triticale were noticed. The pH (H_2O) of most of the soils in the study sites was in a strongly acidic range (4.6–5.5). *Gashena Akayita* of *Banja* District was the most acidic of all and had high levels of exchangeable Al. The limitations of the current coping strategies suggested the need to introduce compatible technologies that would ensure the sustainable management of the soils in the region, by the small-scale farmers there.

Thirty three Released Varieties and selected accessions of tef were evaluated for their tolerance to soil acidity in pot trials. Twenty eight of these were then evaluated under field conditions. The results revealed the presence of significant genetic variability within the test genotypes. Nearly all the test genotypes were highly sensitive to soil acidity and Al-toxicity. However, a local landrace that is widely grown in *Banja,* a District severely affect by soil acidity, consistently outperformed the other genotypes both under pot and field conditions. There were changes in the ranking of the tef genotypes tested under pot and field conditions, which suggested the need to consider other edaphic and climatic factors when breeding for Al-tolerance. Overall, the grain yield of the test genotypes and the tolerant local landrace were less than the national mean yield of tef, identifying the need to develop varieties with better tolerance of acid soils and the associated Al-toxicity, aiming for superior agronomic performances in acid soils.

Twenty-seven tef accessions collected from three regions of Ethiopia that are affected by acid soils were evaluated, together with released breeders' varieties, and selected breeding materials for genetic diversity, using 16 selected and highly polymorphic SSR markers. Analysis of molecular variance (AMOVA) showed highly significant differences ($P<0.001$) among and within populations. Despite the wide geographical separation of the collection sites, 88.5% of the accessions from acid soils were

grouped into two clusters (Clusters II and III) while 90% of the breeding materials and the Released Varieties were grouped into Cluster I. A significant degree of genetic differentiation was observed among the populations. Accessions from the north western Ethiopia exhibited a significant level of variation for most of the genetic diversity parameters. The number of private alleles was significantly higher for tef plants from acid soils than the Released Varieties and the breeding materials the Pair-wise estimates of genetic identity and gene flow showed higher values existed between the Released Varieties and breeding materials.

About 15,000 M_2 seeds were screened under acid soil conditions along with the M_0 mutagenized seeds of the parent variety *Tsedey* and an Al-tolerant local landrace, *Dabo banja*. Twenty one M_2 plants with root lengths of greater than the mean plus standard deviation of the tolerant check were selected and their M_3 progenies were characterized for Al-tolerance and morpho-agronomic traits under greenhouse and field conditions, respectively. There were highly significant differences for Al-tolerance between the M_3 mutant lines and the parent ($P<0.001$); and between the M_3 mutant lines and the sensitive check ($P<0.001$). However, there was no significant difference between the M_3 mutant lines and the tolerant check. The result of the morpho-agronomic characterization revealed the presence of significant differences between the M_3 mutants for 16 of the 20 quantitative traits measured.

Five levels of $AlK(SO_4)_2.12H_2O$ were evaluated (0, 150, 250,350, 450, 550 µM) in order to select the optimal concentration of Al that can most efficiently discriminate between sensitive and tolerant tef genotypes, using a hydroponic growing facility and measuring root lengths. The haematoxylin staining method was also assessed as a tool for the visual evaluation of tef varieties for Al-tolerance using selected test genotypes. There were highly significant differences ($P<0.001$) between the treatments, both for dose of Al and for genotype sensitivity to Al. The maximum differences in relative root length (RRL) (%) and root length (RL) (mm) between the sensitive and the tolerant genotypes were observed at the Al level of 150 µM Al. This concentration efficiently discriminated between 28 test genotypes with different levels of sensitivity to Al-toxicity. A visual assessment of the reactions of two sensitive and two tolerant genotypes to haematoxylin staining using 0, 150 and 250 µM of $AlK(SO_4)_2.12H_2O$ showed differential staining reactions in their roots that were consistent with their prior root growth measurements.

Declaration

I, Ermias Abate Desta, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original work.
2. The thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, picture, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced.
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Signed

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Ermias Abate Desta

As the candidate's supervisors, we agree to the submission of the thesis:

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Prof. Shimelis Hussein (Supervisor)

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Prof. Mark D. Laing (Co-Supervisor)

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Dedication

This PhD study is dedicated

to my late father, Abate Desta Mazengia,

and to my lovely children, Eyerusalem, Meba and Barok

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Thesis Introduction

Background

Tef [*Eragrostis tef* (Zucc.) Trotter] is an allotetraploid (2*n* = 4*x*= 40), herbaceous, annual, cereal crop that is widely produced and consumed in Ethiopia. In terms of area of cultivation, it is the most important crop, followed by maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.). According to the Central Statistical Agency of Ethiopia (CSA) (2015), the area covered by tef during the 2014 cropping season was over 3 million hectares, constituting about 30% of the total area occupied by cereals in the country. Tef production in Ethiopia involves over 6 million rural households (CSA, 2015). Because of its importance in the national diet, the crop is always in demand and fetches better market prices for the farmers than other cereals (Ketema, 1993).

In terms of nutritional composition, tef is equivalent or better than most of the cereals. It contains relatively high levels of the essential amino acids, including lysine (Jansen *et al.*, 1962). According to Ketema (1993), the amino acid composition of tef is excellent and its lysine content is higher than that of all other cereals except rice and oats. The mineral content of tef is also appreciable. Mengesha (1966) found that tef contains more calcium, copper, zinc, aluminium, and barium than wheat, barley, and sorghum. Though there is agreement among various studies that tef is high in iron, it is still unclear whether the iron sources were from the grain *per se* or is a result of contamination with soil during threshing (Almgard, 1963; Mengesha, 1966; Bisrat *et al.*, 1980).

In Ethiopia, tef is used in many ways. The favourite form is '*injera*', a pancake-like, soft and slightly sour bread made of fermented tef flour. *Injera* is traditionally consumed with *wot,* a sauce made of meat, or ground pulses like lentil, faba bean, field pea, broad bean or chickpea. The *injera* are used to wrap up the sauce with no need for spoons or forks. Sometimes tef is used for making porridge, *kitta* (an unleavened bread), local alcoholic drinks such as *tela* (an opaque beer), and *katikala* (a local spirit) (Ketema, 1993). Tef plays a primary role in the daily diet of Ethiopians. As such, its role in food security and the livelihood of Ethiopians who are directly or indirectly involved to its production, processing, marketing, and use is substantial.

Other countries such as Eritrea, the USA, the Netherlands and Israel produce small quantities of tef as a grain crop (Spaenij-Dekking *et al.*, 2005). Currently, tef is gaining growing popularity worldwide as gluten-free healthy food (Spaenij-Dekking *et al.*, 2005). In Europe, tef derived products such as bread, breakfast cereals, breakfast drinks, breakfast bars, performance bars, drinks, pasta, bake-off breads, and cakes are appearing in supermarkets (Turkensteen, 2008).

Besides the grain, tef straw is also an important and highly valued as livestock feed in Ethiopia. Seyoum and Dereje (2001) reported that tef straw is the most important livestock fodder in Ethiopia, and constitutes 27% of the total 14 million tonnes of crop residue produced in the country. They also indicated that crude protein content, *in vitro* digestibility, and energy value of tef straw is higher than that of other cereals. South Africa, India, Pakistan, Uganda, Kenya and Mozambique grow tef mainly as a pasture crop (Assefa *et al.*, 2010).

Tef can be grown from sea level to altitudes of over 3000 m under various rainfall, temperature and soil regimes (Ketema, 1993). However, it yields best within an altitudinal range of 1700-2200 m, an annual rainfall of 750-850 mm or a growing season rainfall of 450-550 mm and a temperature range of 10°C-27°C. In drought prone areas, tef is used as a rescue or emergency crop to replace long maturing crops that fail because of erratic rainfall or damage from pests or diseases (Ketema, 1993). Tef also has excellent tolerance of waterlogging. Tef gives a higher grain yield than wheat by 70% and 106% under poorly drained soil conditions, with and without fertilizer, respectively (Belayneh , 1986). Similarly, Tefera and Ketema (2001) reported that tef performed better than maize, wheat and sorghum (*Sorghum bicolor* L. Moench) under waterlogged conditions. Tef is also preferred for its flexibilie uses in various cropping systems such as double cropping and intercropping (Ketema, 1993).

Constraints to tef production

Improved tef varieties, with recommended agronomic packages, yield as much as 3.2 and 2.6 $t.ha^{-1}$ on research station fields, and on farmers' fields, respectively (Tefera and Ketema, 2001; Assefa *et al.*, 2010). On the other hand, the mean national productivity of tef is about 1.6 $t\ ha^{-1}$. This gap is attributed to various constraints.

Lack of quality seeds of improved varieties; the unavailability of important agricultural inputs such as fertilizers and herbicides, a lack of awareness on the advantages of improved production technologies, and cash shortages are among the major socioeconomic constraints that have contributed to the low adoption of improved tef varieties and their production packages (Tesfaye *et al.*, 2001; Yadeta *et al.*, 2001).

As a crop with an exceptionally small seed, tef is subjected to various constraints. During planting, it needs a well prepared, fine seedbed to germinate, establish, and compete well with weeds. Experimental results have shown that grain yields increase as the number of ploughing events increase from zero to five (Hundera *et al.*, 2001). Gryseels (1998) reported that farmers with no oxen obtained less than half of the yield that farmers with two oxen obtain because of poorly prepared seedbeds and late planting.

Weeds are major biotic constraints of tef in all production areas of Ethiopia. The problem is mainly associated with the poor competitive capacity of the crop and the difficulty in establishing uniform stands because of the small size of tef seeds. The intensity of the problem varies with the soil and climatic factors and the farmers' production practices. Fisehaye and Tadele (2001) reported about 64 plant species in 60 genera and 24 plant families as weeds on tef. These authors indicated that the parasitic weed *Striga hermonthica* (Del.) Benth, *Parthenium hysterophororulus* L.*, Convolvulus arvensis* L.*,* and *Cyperus rotundus* L. to be the most serious weeds*.* National yield loss assessments associated with weeds on tef have varied between 23-65%. Farmers use hand weeding, herbicide, higher seed rates, and increased numbers of ploughing operations to overcome the problem of weeds (Fisehaye and Tadele, 2001; Yadeta *et al.*, 2001).

Compared to other crops, insect pests and diseases are of minor importance on tef in the major production areas. Among 36 insect pest species known to attack tef the most important nationally are the Wollo bush-cricket *(Decticoides brevipennis* Ragge), red tef worm *(Mentaxya ignicollis* Walker), shootfly *(Hylemya arambourgi* Seguy*),* tef fly [*Delia arambourgi* (Seguy)], black tef beetle *(Erlagerius niger* Weise), grasshopers, and termites (Ketema, 1993; Chichaybelu *et al.*, 2001).

So far, over 24 fungi and two nematode species have been recorded to cause disease on tef. Among the fungal diseases, Stewart and Dagnachew (1967) as cited by Ketema (1993) reported that tef rust caused by *Uromyces eragrustidis* Tracy and head smudge caused by *Helminthosporium miyakei* Nisikado are the most important diseases of tef, but mainly in minor production areas. The two nematode species belonged to the genus *Paratylenchus* and were of minor importance (Eshetu, 1986).

Lodging is the most important constraint of tef production in Ethiopia. Ketema (1993) estimated mean grain yield loss due to lodging under natural conditions at 17% with a maximum loss of 27%. Teklu and Tefera (2005) reported tef yields of 4.6 $t.ha^{-1}$ for tef when supported with nets relative to yields of 2.4-3.4 $t.ha^{-1}$ when grown under natural conditions. Lodging affects tef production in several ways. It prevents the crop from ripening properly, and it often results in mouldy panicles, inferior seed quality and sprouting seeds on the panicle (van Delden *et al.*, 2010). Consequently, it reduces seed vigour and the germination potential of the seed. Lodging also decreases productivity of the crop by hindering optimal use of external inputs such as nitrogen (Assefa *et al.*, 2010). Thus, potential yield increments from Released Varieties is sacrificed because of limited use of production inputs such as mineral fertilizers. Attempts made to curb lodging through breeding have not been successful due to the lack of adequate natural variation for lodging resistance, and negative associations between lodging resistance traits with productivity enhancing traits such as plant height, panicle length, panicle form, grain and shoot biomass yield (Assefa *et al.*, 2010).

Soil acidity, and poor soil fertility, are among the major abiotic constraints on tef production (Dubale, 2001; Tadesse, 2001; Holden and Shiferaw, 2004; IFPRI, 2010). Mineral fertilizer recommendations have been provided to reduce the problems of poor soil fertility. However, optimal use of fertilizers is constrained by lodging losses which are caused by luxurious growth of tef plants (Assefa *et al.*, 2010). An additional problem is that tef crops respond poorly to applied fertilizers in acid soils (Mamo and Killham, 1987).

Soil acidity is one of the major production constraints of crops worldwide. Over 40% of the arable lands of our world have problems of soil acidity, and 22% of the arable lands of Africa have soil acidity (pH < 5.5 in the surface layer) (von Uexk¨ull and Mutert,

1995). About 67% of the world's acid soils have crop production constraint associated with Al-toxicity (Eswaran *et al.*, 1997).

The most important cause of soil acidity is the leaching of basic cations to the lower profiles of the soil by percolating rain water. The acidifying effect of acid forming nitrogen fertilizers, poor nutrient recycling and the continuous removal of basic cation through harvested crops, runoff loss and acid rain also contribute to the development of soil acidity and Al-toxicity.

Leaching leaves the soil with the acidic cations, aluminium, manganese and hydrogen. Accumulation of aluminium causes toxicity that results in severe restriction of root growth. Consequently, absorption of minerals and water is affected. The resultant low soil pH also decreases the availability of important plant nutrients such as phosphorous, nitrogen, potassium, calcium, magnesium, sulphur, zinc and molybdenum (Rao *et al.*, 1993). Furthermore, crops do not respond to fertilization with nitrogen because of the fixation or unavailability of phosphorous in acid clay complexes. Mamo and Killham (1987) reported the poor response of tef to fertilizer applications when grown in acid soils.

Due to restriction on root development, the vulnerability of crops to droughts increases (Little, 1989; Foy, 1992). This is particularly important because most acid soils have inherently low water holding capacity, being highly leached soils (Little, 1989).

The overall effects of Al-toxicity are stunted growth and low productivity (Rao *et al.*, 1993). Growth of several tropical crops in areas with acid soils are reduced by 50% or more when compared to plants grown on limed soil (Kamprath, 1984). Gallardo *et al.* (1999) also reported about 50% reduction in grain yields due to Al-toxicity. In wheat, Tang *et al.* (2001) found that liming increased shoot weight and grain yield by 60% and head number by 32%. Such yield increases are highly correlated with decreases in exchangeable Al as a result of liming.

Problem statement

Aluminium toxicity and other acidity related soil fertility problems are among the major constraints affecting crop production in Ethiopia (Dubale, 2001; IFPRI, 2010). The problem is widespread in the western, southern, south-western and the north-western part of the country, where reliable rainfall is available. This is in contrast with the

unpredictable and often inadequate rainfall that falls on the eastern parts of the country.

Liming is the most common and widely used method to ameliorate the impact of Al-toxicity in acid soils (Rao *et al.*, 1993). In the tropics, utilization of lime is constrained by various factors. Due to sub-surface acidity and the strong buffering capacity of tropical acid soils, large quantities of lime are needed to ameliorate acid soils in these areas (Rao *et al.*, 1993; The *et al.*, 2006). For most of the resource poor farmers in the tropics, local unavailability, and the high costs of lime and its transport costs are prohibitive. The inherent slow mobility of lime in the soil, and the difficulties of mechanical incorporation into the sub-soil without large tractors are also problems for small-scale farmers dealing with sub-surface acidity. Runoff pollution and the adverse effects of lime on calcifuge crops in rotation system are negative effects of lime applications (Wang *et al.*, 2006).

The use of organic matter in the form of manure and compost can significantly reduce soil acidity (Wong and Swift, 2003). Several organic compounds released from the decomposition of organic matter are efficient in detoxifying Al^{3+} by forming various complexes with it (Haynes and Mokolobate, 2001). In addition, regular application of organic matter increases soil pH, and helps in the conversion of toxic species of Al to non-toxic and insoluble hydroxyl-Al compounds. However, the regular and high volume application of organic matter to the highly weathered soils of the tropics is constrained by several factors. In countries such as Ethiopia, animal manure and crop residues have many other uses, including as fuel, animal feed, and construction material. Therefore, there is little retention of organic matter on fields after harvest (Schlede, 1989; IFPRI, 2010).

Di-ammonium phosphate (DAP) and urea are the mineral fertilizers almost exclusively applied in Ethiopia, on all soil types and in all agroecologies of the country (Abebe, 2007). These fertilizers are acid-forming fertilizers and their use in areas with acid soils is known to aggravate the level of acidity (Barak *et al.*, 1997; Bolan and Hedley, 2003). Currently, with the objective of improving crop productivity per unit area, the enhanced use of these fertilizers is being promoted by the national agricultural extension services across the country, irrespective of the negative consequences in areas with acid soils.

As a consequence of high human and animal population pressures, farmers in cereal dominated production areas have abandoned traditional fertility management practices such as fallowing. Crop rotation is not practiced due to land shortages, and the unsuitability of acid soils for rotation crops. Overall, the balanced use of external inputs is very low, and soil erosion is a widespread problem. All these factors ensure that outflows of nutrients from the system are common. Consequently, in some areas of Ethiopia affected by acid soils, farmers grow crops such as oat (*Avena sativa* L.), triticale (x *Triticosecale* Wittm. Ex A. Camus) and white lupin (*Lupinus albus* L.) that are adapted to acid soils, in order to ensure their household food security. These crops, however, do not have a good market demand and value compared to other popular cereals.

Another problem is that areas which were previously under forest, woodland and savannah are being converted to fields in order to increase production of food, industrial and biofuel crops. These virgin soils, especially in the southern, south western and north western parts of the country, have a strong tendency towards acidification because of the parent material of the soil, the low buffering capacity of the soils (high acid saturation) and the conducive climatic factors (Abebe, 2007).

Hence, if the goal of food security across the whole country through increased productivity and production of crops is to be achieved, then the acid soils need to be managed through a system of integrated soil management that is based on a number of interventions. From this perspective, the breeding and release of Al-tolerant varieties would be a socially, economically, and technically achievable way to assist small-scale farmers of developing countries like Ethiopia to grow their crops in acid soils. The integration of tolerant crop varieties, lime and organic fertilizers would have a synergistic effect, resulting in increased crop productivity, and in sustainable soil health.

Currently, the breeding of crops, including tef, for tolerance of low soil pH or Al toxicity has not yet received adequate research attention. Most of the released tef varieties were bred primarily for optimal growing conditions (Assefa *et al.*, 2010). A failure to target specific production constraints has been implicated in the high genotype by environment interactions that have been documented in several tef experiments, and the overall decline in genetic gains from tef breeding programmes (Assefa *et al.*, 2010).

In order to undertake long-term breeding for Al-tolerance in tef, a number of pre-breeding activities have to be accomplished first. These include an appraisal of the importance of soil acidity, farmers' preferences and selection criteria for tef varieties, an assessment of genetic variability in tef for tolerance of soil acidity and Al-toxicity, and the development of appropriate phenotyping techniques

The overall goal of this study was to improve food security and income of tef farmers in areas with acid soils by enhancing the productivity of tef through the development of high yielding and Al-tolerant varieties. In order to achieve this goal, several experiments were conducted with the following specific objectives.

Objectives:

1. To assess farmers' perceptions of soil acidity and Al-toxicity in areas with acid soils in north western Ethiopia, and their coping strategies;
2. To characterize the reactions of a diverse collection of tef varieties and related species, including released tef varieties, to soil acidity and Al-toxicity;
3. To determine the extent of genetic diversity among tef germplasm collected from areas of Ethiopia with acid soils, using SSR markers;
4. To screen and characterize EMS-induced mutants of tef for tolerance to Al-toxicity and other important agronomic traits;
5. To develop a high-throughput, hydroponics system as a phenotyping platform to screen for tolerance to Al-toxicity in tef, using root measurements and a haematoxylin assay to quantify varietal responses to Al toxicity.

Outline of thesis

This thesis consists of seven chapters including a literature review, a participatory rural appraisal (PRA) and five experimental chapters (see outline below). The referencing system used in this thesis is based on the referencing style of the Journal of Crop Science. The thesis is in the form of discrete research chapters, each following the format of a stand-alone research paper (whether or not the chapter has already been published). This is the dominant thesis format adopted by the University of KwaZulu-Natal.

Chapter 1, titled "Aluminium toxicity tolerance in cereals: mechanisms, genetic control and breeding methods", was published in the African Journal of Agricultural Research

Vol. 8(9), pp. 711-722, doi: 10.5897/AJARx12.003." Chapter 3, entitled "Quantitative responses of tef [*Eragrostis tef* (Zucc.) Trotter] and weeping lovegrass [*Eragrostis curvula* (Schrad.) Nees] varieties to acid soil" was published in the Australian Journal of Crop Sciences 7(12):1854-1860 (2013) ISSN: 1835-2707.

Chapter	Title
-	Thesis introduction
1	A review of the Literature
2	Soil acidity: Importance, assessment of perceived causes and indicators, coping strategies and implications in cereal based mixed-farming system of north western Ethiopia
3	Preliminary investigation on presence of genetic variability for soil acidity in tef [*Eragrostis tef* (Zucc.) Trotter]
4	Response of selected tef [*Eragrostis tef* (Zucc.) Trotter] genotypes to soil acidity under pot and field experiments
5	Evaluating the genetic diversity of tef [*Eragrostis tef* (Zucc.) Trotter] accessions collected from sites in Ethiopia with acid soils, using simple sequence repeats (SSR) markers
6	Isolation and characterization of ethyl methane sulphonate (EMS) induced mutants of tef [*Eragrostis tef* (Zucc.) Trotter] for aluminium tolerance and morpho-agronomic traits
7	Development of a hydroponic phenotyping platform to assess aluminium tolerance in tef [*Eragrostis tef* (Zucc.) Trotter] genotypes
8	Overview of major research findings and their implications

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CHAPTER 1

A review of the Literature[1]

Abstract

Aluminium toxicity is a major crop production constraint of acid soils with a pH of below 5.0. About 67% of the world's acid soils have crop production constraints associated with Al-toxicity. In Ethiopia, about 13.2% of the total land area is strongly to moderately acidic (pH < 5.5). Management of acid soils in this country involves application of mineral fertilizers, lime, manure and compost. However, utilization of these options is constrained by various technical and socio-economic factors. Existence of natural factors that favour the development of soil acidity and the shortcomings of the existing management options suggest that soil acidity will remain as one of the most important challenges affecting Ethiopian agriculture. Hence, the development of complementary and acceptable management options is of paramount importance. Due to their wide area of production, cereals are the crops most affected by soil acidity and Al-toxicity. In regions of the world affected by acid soils, use of Al-tolerant crop varieties and non-genetic management options such as liming are common practices. In Ethiopia, breeding for tolerance to soil acidity has not yet received adequate research attention. Consequently, for crops such as tef, which are of little economic importance beyond Ethiopia, there is no or little information available on the reaction of the crop to soil acidity. Hence, parallel studies on other cereals are presented to assist in developing a framework for the breeding of tef with tolerance of soil acidity and Al-toxicity. Thus, this article reviews the basic information available on Al-toxicity, tolerance mechanisms, screening methods and the prospects of molecular marker assisted selection for Al tolerance in cereals.

Key words: Al-toxicity, Al-tolerance, cereals, soil acidity

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1.1 Introduction

The global population is projected to reach 9 billion by the year 2050. The world will require 70 to 100% more food to feed this population (FAO, 2009). Mean annual increments of 44 million metric tons per year will be needed in the coming years to meet this demand. And this mean annual increment is more than a 38% increase over the historical increase in global food production (Tester and Langridge, 2010). Currently, more than 50% of world's daily calorie requirement or about 80% of calories in the poorest countries of the world are directly derived from cereal grains (Awika, 2011). However, the mean productivity of major cereals in the globe is substantially lower than the yield that can be obtained under ideal conditions Hence, meeting the projected global food demand needs to focus on enhancing the productivity of cereals in the low-yielding environments of the world (Tester and Langridge, 2010; Godfray *et al.*, 2010).

In Ethiopia, cereals are the predominant staple food crops of the population. They accounted for about 10.14 million ha or over 80.78% of the total area cultivated by grain crops (cereals, pulses and oil crops) in small-scale holdings (CSA, 2015). In terms of volume of production, cereals contributed over 23.6 million tonnes or 87.31% of the total grain produced in the country (CSA, 2015) (Table 1.1). However, the national productivity of these crops is lower than the global mean (Table 1.1). In addition to technical issues, socio-economic and market related problems, environmental constraints such soil acidity make a significant contribution to the low national mean productivity.

Soil acidity is one of the major crop production constraints worldwide. Over 40 % of the arable land in the world has problems of soil acidity (pH< 5.5 in surface layer) and 22% of the arable soils in Africa have soil acidity problems (von Uexk¨ull and Mutert, 1995). About 67% of the world's acid soils have crop production constraints associated with Al-toxicity (Eswaran *et al.*, 1997). In Ethiopia, the soils of about 13.2% of the total land area are estimated to be moderately to strongly acidic (pH < 5.5) (Schlede, 1989).

The overall effects of soil acidity are stunted growth, poor responses to applied fertilizers and vulnerability to drought. Consequently they are low potential soils. Al and Mn toxicities and deficiencies of macro- and micro-nutrients are major causes of these effects. Since Al dissolves at pH values below 5-5.2, all of the soils in Ethiopia

that fall into the moderately and highly acidic classes (pH<5.5) are likely to have Al-toxicity. The paradox of the soil acidity problem in Ethiopia is that most of the acid soils are found in the high rainfall areas, which are falsely presumed to be high potential production zones for cereals and perennial cash crops (Abebe, 2007; IFPRI, 2010).

Applications of mineral fertilizers, lime, compost and manure are the official components of acid soil management in Ethiopia. However, utilization of these options is constrained by various technical and socio-economic constraints. The existence of natural factors that enhance the development of soil acidity, and the shortcomings of the existing management options, suggest that soil acidity will remain as one of the most important challenges affecting Ethiopian agriculture. Hence, the development of complementary and acceptable management options is of paramount importance in order to increase productivity of crops in such environments.

In areas of the world prone to acid soils, utilization of crop varieties tolerant of Al-toxicity; along with other management methods, are common practices. In Ethiopia, the development of varieties adapted to specific marginal growing environments, such as acid soils, has not yet received adequate research attention. The lack of such strategies is considered to have contributed to declining genetic gains for nationally important crops such as tef (Assefa *et al.*, 2010).

In the world, breeding crops for tolerance to Al-toxicity has received strong research patronage for several decades. Basic studies have been conducted on mechanism of tolerance to Al-toxicity, genetic control of tolerance to Al-toxicity and screening methods for all the globally important crops. Conventional and molecular breeding methods have also been applied to develop Al-tolerant crop species. This chapter reviews basic information available on Al-toxicity and advances in breeding for tolerance to Al-toxicity in cereals with the objective of using this information as a basis for the breeding of tef for tolerance to Al-toxicity, as documented in the subsequent experimental chapters.

Table 1.1. Area, production and productivity of cereal crops and other crops in Ethiopia during 2014 cropping season under small holders (CSA, 2015).

Crops	Area (ha)	% of total grain area	Yield (tons)	% of total grain yield	National mean productivity (tons.ha^{-1})	*Global mean productivity (tons.ha^{-1})
Cereals	**10152015.05**	**80.76**	**23607662.44**	**87.30**		
Tef	3016062.55	24.03	4750657.28	17.58	1.58	-
Barley	993938.74	7.92	1953384.78	7.23	1.97	2.77
Wheat	1663845.63	13.26	4231588.72	15.66	2.54	2.88
Maize	2114876.10	16.78	7234955.10	26.74	3.43	5.16
Sorghum	1834650.10	14.57	4339134.26	16.03	2.37	1.5
Finger millet	453909.38	3.62	915314.52	3.39	2.01	-
Oats	27899.64	0.22	50805.93	0.19	1.82	-
Rice.	46832.21	0.37	131821.85	0.49	2.82	4.20
Pulses	**1558422.02**	**12.42**	**2671834.45**	**9.89**		
Oilseeds	**855762.91**	**6.82**	**760099.32**	**2.81**		
Total grains	**12566239.98**	**100**	**27039604.80**	**100**		

*source:Awika (2011)

1.2 Development of aluminium toxicity: An overview

Aluminium is the most abundant metal in Earth's crust comprising 7% of its mass. It is also the third most common element in the earth's crust (Vitorello *et al.*, 2005). In soils, Al mostly exists as a structural constituent of primary and secondary aluminosilicate minerals (Delhaize and Ryan, 1995; Miyasaka *et al.*, 2007).

Acidity and aluminium toxicity develops as a consequence of leaching of basic cations in soils of high rain fall areas that have high drainage. The acidifying effect of acid forming nitrogen fertilizers, poor nutrient recycling and the continuous removal of soil cations through harvested crops, runoff losses and acid rain also contribute to the development of soil acidity (Rao *et al.*, 1993; von Uexk¨ull and Mutert, 1995; Barak *et al.*, 1997). As the soil becomes acidic, the silicon from aluminosilicate minerals are leached, leaving Al in solid forms such as aluminium oxyhydroxides, including boehmite and gibbsite. These forms release the phytotoxic aluminium species, Al^{3+} (also represented as $Al(H_2O)_6^{3+}$) into the soil solution when the pH goes below 5.0 (Abebe, 2007; Miyasaka *et al.*, 2007).

Negatively charged clay particles can remove Al^{3+} from the soil solution and therefore, they can reduce its toxicity to plants. Similarly, organic matter has many negatively charged carboxyl ($-COO^-$) functional groups that can remove Al^{3+} from soil solutions by forming organic complexes. Other complexing inorganic anions such as SO_4^{2-}, PO_4^{3+} and organic anions such as citrate, malate, and oxalate can have the same effect (Delhaize and Ryan, 1995; Miyasaka *et al.*, 2007). Concentration of cations at a given pH also considerably affects the toxicity of Al^{3+}. For instance, when the concentration of Ca^{2+} is optimal for a plant's requirement, then the toxic effect of Al^{3+} is reduced (Wang *et al.*, 2006).

1.3 Mechanisms of aluminium toxicity

Despite its abundance in the earth's crust, Al is not known to have a natural role in the physiology of any living organisms (Vitorello *et al.*, 2005). Knowledge on the absorption of the phytotoxic forms of Al^{3+} is also limited. The toxic trivalent species cannot pass through the plasma membrane. Hence, it is hypothesised that it enters the root system either by endocytosis or via the calcium channels of the plasma membrane (Miyasaka *et al.*, 2007).

The transition region of root apex is the primary target of Al toxicity in plants. Kochian *et al.* (2005) and Miyasaka *et al.* (2007), in their comprehensive review of the subject, suggested that aluminium toxicity could potentially result from interaction of aluminium with the apoplast (cell wall), plasma membrane, symplastic (cytosol) targets, signal transduction pathways, the root cytoskeleton and DNA.

1.4 Symptoms and effects of aluminium toxicity

The most important short-term symptom of Al toxicity is the inhibition of root growth, which is expressed within a few minutes to a few hours after exposure to micromolar concentrations of Al (Barcelo and Poschenrieder, 2002). Root inhibition can be exhibited by primary and lateral root apexes, and such roots become thick and develop brown colour (Vitorello *et al.*, 2005; Wang *et al.*, 2006; Claudio *et al.*, 2008). The distal transition zone (DTZ) of root tip, where the cells switch from cell division to cell elongation, is the most sensitive part of roots (Barcelo and Poschenrieder, 2002; Miyasaka *et al.*, 2007).

Callose formation and lignin deposition in cortical cells of roots are reported to be one of early symptoms of Al toxicity in various plant species (Miyasaka *et al.*, 2007).

Reduced branching of fine roots, suppression of root hair development and abnormal root morphology are consequences of long term exposure of Al-sensitive plants to toxic concentration of Al (Vitorello *et al.*, 2005; Miyasaka *et al.*, 2007). These effects directly impact upon nutrient uptake as well as water absorption. Consequently, deficiencies of calcium, magnesium, potassium, iron, molybdenum and phosphorus are common symptoms in plants grown in soils with Al toxicity problems (Vitorello *et al.*, 2005; Wang *et al.*, 2006; Miyasaka *et al.*, 2007). Excess Al in soil also has a negative effect on the nitrogen fixing capacity of symbionts in legumes, and this is associated with the Al sensitivity of the rhizobial strains, resulting in reduced nodulation (Miyasaka *et al.*, 2007). The inhibitory effect of Al on root development decreases tolerance of plants to drought and use of subsoil nutrients (Little, 1989; Foy, 1992; Carver and Ownby, 1995; Haynes and Mokolobate, 2001).

Suppression of photosynthetic capacity is associated to cellular and ultrastructural modifications in leaves; reduced stomatal opening and CO_2 assimilation; reduced chlorophyll concentration; chlorosis and leaf necrosis are also effects of Al-toxicity (Vitorello *et al.*, 2005; Moreno-Mateos *et al.*, 2007; Chen *et al.*, 2010). Some of the indirect effects of Al toxicity are increased susceptibility of stressed plants to diseases (Little, 1989).

Consequently, the outcome of Al toxicity is significantly expressed on biomass and grain yield of crops. Growth of several tropical crops in areas with acid soils that had soil aluminium saturation levels of greater than 60% was reduced by 50% or more when compared to plants grown on limed soil (Kamprath, 1984). Gallardo *et al.* (1999) also reported 50% and 30% reductions of grain yield in Al sensitive and tolerant varieties, respectively. In a study on wheat, liming increased shoot weight and grain yield of Al-sensitive genotypes by 60% and head number by 32% (Tang *et al.*, 2001). Such yield increases are highly correlated with reduced levels of exchangeable Al as a result of liming.

1.5 Breeding for tolerance to Al toxicity

Lime is the most common and widely used method to ameliorate the impact of Al-toxicity in acid soils of temperate regions (such as Europe and North America) (Rao *et al.*, 1993). In these areas, soil acidity develops in surface soils mainly as a

consequence of the heavy use of mineral fertilizers, and from environmental pollution (Rao *et al.*, 1993).

In the tropics, significant yield increase may result from the appropriate application of lime. However, due to sub-soil acidity, and their strong buffering capacity of acid soils, such soils need substantial doses of lime to neutralize the acidity (Rao *et al.*, 1993; The *et al.*, 2006). Most of resource poor farmers in the tropics are constrained by the local unavailability of lime, its high cost, and the costs of transporting a bulky product in the quantities that are needed. Furthermore, these farmers lack the appropriate technology for deep mechanical incorporation, which combines with the inherently slow movement of lime into soils, and especially the acidic sub-soils. Consequently, root development of acid sensitive crops is restricted to the surface soil, leaving these crops vulnerable to even minor droughts (Little, 1989; Foy, 1992). This is particularly important because many acid soils have inherently low water holding capacity (Little, 1989; Haynes and Mokolobate, 2001). Runoff pollution and the adverse effect of lime on calcifuge crops in rotation systems are negative side effects of lime applications (Wang *et al.*, 2006).

The use of organic matter in the form of manure and compost can significantly reduce soil acidity (Wong and Swift, 2003). Many organic compounds are released or synthesized during the decomposition of organic matter by soil microorganisms. Among these, soluble humic molecules and low molecular weight aliphatic acids are efficient in detoxifying Al^{3+} by forming various complexes (Haynes and Mokolobate, 2001). In addition, the regular application of organic matter increases soil pH. Under higher pH the toxic species of Al are converted to non-toxic and insoluble hydroxyl-Al compounds. Application of organic matter also improves the availability of deficient soil nutrients such as phosphorus. Use of organic matter seems an applicable strategy to resource poor farmers of the tropics who cannot afford large quantities of lime and fertilizers. However, in countries like Ethiopia, animal manure and crop residues have many uses, including their use as fuel, animal feed, and construction material. Therefore, regular applications of organic matter to acid soils are not common (Schlede, 1989; IFPRI, 2010).

Di-ammonium phosphate (DAP) and urea are the mineral fertilizers exclusively used on all soil types and agro-ecologies in Ethiopia (Abebe, 2007). Assimilation of these fertilizers into roots produces protons are excreted into the external medium,

increasing soil acidity (Marschner, 1995; Barak *et al.*, 1997; Bolan and Hedley, 2003). Hence, these fertilizers are classified as acid-forming fertilizers. Cautious use of these fertilizers in areas with acid soils involves the concurrent application of acid-equivalent quantities of lime that can immediately neutralize acidity as it is released (Bolan and Hedley, 2003). Accordingly, the acidity equivalent or the number of parts of pure lime (calcium carbonate) required to neutralize the acidity caused by 100 parts of DAP and Urea is 74 and 79, respectively. For these two fertilizers, DAP and urea, the number of years required to decrease the pH by one unit varies between 10-33 and 25-78 years, respectively, depending on the buffering capacity of the soil to change in pH (Bolan and Hedley, 2003). Currently, with the objective of improving crop productivity per unit area and bridging the existing yield gap between potential and actual yields, the extension services of the country are promoting the exclusive use of these fertilizers, without concurrent applications of lime The outcome of this practice will be that the less acidic soils will systematically be converted to strongly acidic soils.

As consequence of high human and animal population pressures, farmers in cereal dominated production areas have abandoned traditional fertility management practices such as fallowing. Crop rotation lesson longer widely practiced due to land shortage and the unsuitability of acid soils for Al-intolerant rotation crops. Contending uses of crop residue and animal manure has restricted farmers from using these resources to replenish soil fertility. Overall, utilization of external inputs is unbalanced and very low. Soil erosion is rampant. All these factors combine to ensure that a substantial outflow of mineral nutrients from the soil system is very common. Consequently, in some areas of Ethiopia affected by acid soils, farmers have changed to growing crops such as oat, triticale, white lupine that are adapted to acid soils, in order to ensure their household food security. However, these crops, do not have a good market demand or value compared to popular cereal crops, especially tef.

Furthermore, areas that were previously under forest, woodland and savannah are being converted into crop production fields in order to increase national production of food, industrial and biofuel crops. These soils, specifically the ones from the southern, south western and north western parts of the country have a strong tendency to become acid because of the parent soils, and the favourable climatic factors (Abebe, 2007).

Hence, the goal of food security through increased productivity and production needs to employ sound and integrated methods to manage acid soils. From this perspective, the breeding and release of Al-tolerant varieties would be socially, economically and technically acceptable, and environmentally friendly for small-scale farmers of developing countries like Ethiopia. The combined uses of tolerant crop varieties, lime and organic fertilizers have a synergistic effect that will result in the increased productivity and sustainable health of acid soils. For instance, The *et al.* (2006) reported that maize varieties tolerant of acid soils gave 61% higher grain yields than Al-sensitive varieties. With lime treatments, yield increases of 208% and 82% were obtained for Al-sensitive and Al-tolerant varieties, respectively.

1.6 Mechanisms of aluminium tolerance in cereals

Globally, the breeding and utilization of Al-tolerant varieties has been used to complement to liming and other non-genetic management options in the production of globally important cereals in acid soils. The exclusion of Al from root apices and the detoxification of Al in the root and shoot symplasm are two known mechanisms of Al tolerance mechanisms in plants. In cereals and grass species, the exclusion mechanism is the most common mechanism.

1.6.1 Exclusion mechanism

Among cereals, exudation of an organic acid, malic acid, in Al tolerant genotypes was first reported on wheat (Delhaize *et al.*, 1993b; Basu *et al.*, 1994; Ryan *et al.*, 1995b). More recently, a second mechanism of Al-tolerance that involves the efflux of citrate has been reported (Ryan *et al.*, 2009). Exclusion mechanisms involved in important cereal crops are summarized in Table 1.2. Organic acids are exuded from the first few millimetres of root apices (Rincon and Gonzales, 1992; Delhaize *et al.*, 1993b). Chelation of the Al ion with the organic acids in the rhizosphere prevents the Al^{3+} from binding to the negatively charged sites of the cell wall and the plasma membrane of tolerant varieties (Miyasaka *et al.*, 1991; Delhaize *et al.*, 1993b; Kochian *et al.*, 2005). In contrast, Al-sensitive genotypes accumulate Al in their root apices. Exudation of organic acids is associated with the activation of a trans-membrane channel upon exposure of the roots to toxic Al concentrations. Organic acids that normally exist as anions in the cytoplasm are released into the root environment following activation of the trans-membrane channel.

1.6.2 Internal detoxification

Compared to the exclusion mechanism, internal detoxification is a less common mechanism in cereals. In rice, the Al-specific expression of the *OsALS1* gene localized in the tonoplast of root cells is reported to sequestrate Al into vacuoles as an internal detoxification mechanism (Huang *et al.*, 2012). Earlier, a similar mechanism was found to operate in Al-tolerant barley and wheat varieties (Taylor *et al.*, 1997). A rapid, Al-induced increase in Mg concentration in the cytosol also increases tolerance of rice to Al (Chen *et al.*, 2012). These authors suggested that an increase in Mg concentration in the cytosol prevents Al binding to enzymes and other cellular components. Masking aluminium binding sites through the modification of cell-wall composition of root cells has also been hypothesized (Huang *et al.*, 2009) (See genetic control below).

Table 1.2. Exclusion mechanisms involved in Al-tolerance of various cereals

Crop	Exudation	Reference
Wheat	Malate	(Delhaize *et al.*, 1993a; Basu *et al.*, 1994; Ryan *et al.*, 1995a)
	Citrate	(Ryan *et al.*, 2009)
	Phosphate and malate	(Didier *et al.*, 1996; Pellet *et al.*, 1997)
	Polypeptides	(Basu *et al.*, 1997; Basu *et al.*, 1999)
Barley	Citrate	(Zhao *et al.*, 2003)
	Phosphate	(Wang *et al.*, 2006)
Rice	Citrate	(Ishikawa *et al.*, 2000; Yokosho *et al.*, 2011)
Sorghum	Citrate	(Magalhaes, 2002)
Rye	Citrate	(Li *et al.*, 2000; Ma *et al.*, 2002a)
Triticale	Citrate and malate	(Li *et al.*, 2000; Ma *et al.*, 2002a)
Maize	Citrate and malate	(Renato and Paulo, 1997)
	Phenolic compounds	(Kidd *et al.*, 2001)

Plants that can accumulate silicon in their roots can release the silicon to detoxify aluminium by forming aluminosilicate compounds in the root apoplast (Cocker *et al.*, 1998). In cereals, this mechanism has been reported in sorghum, where Al and silicon are complexed in the outer wall of the endodermis of roots (Hodson and Sangster, 1993). Suicidal death of cells affected by Al is also reported as a detoxification mechanism in wheat (Delisle *et al.*, 2001). Hypersensitive reactions of cells is a common mechanism in plant defence against pathogens.

1.7 Genetic control of Al-tolerance in cereals

A comparative mapping study found extensive synteny or co-linearity among the genomes of rice, wheat, barley, rye, oat, maize and sorghum (Devos and Gale, 2000).

Genetic control of Al-tolerance in cereals is mainly associated with genes that control protein families linked to membrane transport (Table1.3). In a diverse range of wheat genotypes, a major aluminium tolerance gene at Alt_{BH} in wheat, *TaALMT1*, ***T****riticum* ***a****estivum* **A**luminium activated **M**alate **T**ransporter1, encodes for an Al-activated plasma membrane protein that allows for the efflux of malate from root apices upon exposure to Al (Sasaki *et al.*, 2004; Raman *et al.*, 2005b). This gene was mapped to Chromosome 4DL using 'Chinese spring' deletion lines and its absence resulted in the loss of Al-tolerance and malate exudation (Raman *et al.*, 2005b). Another mechanism of Al-tolerance was found in Brazilian wheat cultivars that involves the efflux of citrate from root apices. The controlling gene, which resides on Chromosome 4BL, has been identified (Ryan *et al.*, 2009). These authors indicated that the citrate efflux is controlled by a single gene, which could explain 50% of the phenotypic variation in citrate efflux. However, unlike the *TaALMT1*, this gene belongs to a gene encoding a **m**ultidrug **a**nd **t**oxic compound **e**xtrusion protein and was designated as *TaMATE1* (Ryan *et al.*, 2009).

In barley, Echart *et al.* (2002) found that an F2 generation analysed with haematoxylin staining followed the Mendel's segregation ratio of 3:1 for Al toxicity tolerant to sensitive plants, revealing the fact that the trait is controlled by single dominant gene at the *Alp* locus, which is located on the long arm of Chromosome 4H. This locus is associated with the Al-induced efflux of citrate from the root apices of tolerant barley encoded by a multidrug and toxic compound extrusion (*HvMATE*) protein (Wang *et al.*, 2007). Quantitative trait loci that explained 50% of the phenotypic variation werealso associated with the same chromosomal location (Ma *et al.*, 2004). Similarly, Raman *et al.* (2005a) identified QTLs for root elongation under aluminium stress on 3H, 4H, 5H and 6H chromosomal locations.

In rye, four independent loci, *Alt1, Alt2, Alt3 and Alt4,* located on chromosome arms 6RS, 3RS, 4RL and 7RS, are known to confer tolerance to Al-toxicity (Matos *et al.*, 2007). Specifically, the *Alt4* locus contained cluster of genes homologous to the Al-activated malate transporter (*TaALMT1*) (Collins *et al.*, 2008). Tolerant and sensitive rye genotypes contained five and two genes of the clusters at the locus, respectively. Out of these, two *ScALMT1*-M39.2 and one *ScALMT1*-M77 genes were highly expressed in the root tip (Collins *et al.*, 2008). Silva-Navas *et al.* (2012) subsequently located a gene coding for a multidrug and toxic compound extrusion protein family

(*ScMATE*), an aluminium-activated citrate transporter, at the same chromosomal location, the 7RS chromosome arm, 25 cM away from the *ScALMT1*.

Magalhaes *et al.* (2007) identified a gene encoding for a member of the multidrug and toxic compound extrusion family *(SbMATE),* which is the responsible gene for the major sorghum aluminium tolerance locus, *AltSb*. They also suggested that polymorphisms in the regulatory regions of *AltSb* are likely to contribute to large allelic effects, acting to increase *AltSb* expression in the root apices of tolerant genotypes. Earlier, Caniato *et al.* (2007) suggested the possibility of the presence of additive or co-dominant effects of different loci that would explain the occurrence of transgressive segregation observed in some tolerant lines.

In maize, an Al-activated efflux of citrate from roots is well characterized and is the most important mechanism of Al tolerance (Renato and Paulo, 1997). The responsible gene is a member of the multidrug and toxin extrusion family (Maron *et al.*, 2008). Maron *et al.* (2010) identified two members of the *MATE* family, *ZmMATE1* and *ZmMATE2*, which co-localized with two independent Al-tolerance QTLs. The authors clearly showed the association of *ZmMATE1* with up-regulation of citrate release at the root tips of tolerant varieties upon exposure to Al. In their most recent study, the authors indicated that a higher copy number of the gene encoding for *ZmMATE1* was responsible for quantitative tolerance to Al toxicity (Maron *et al.*, 2013). *ZmNrat1*, a maize homolog to the rice *OsNrat1*, described below, was also found to have a role in maize Al tolerance (Guimaraes *et al.*, 2014).

In rice, several QTLs have been identified that contribute to phenotypic variations for Al-tolerance (Ma *et al.*, 2002b; Nguyen *et al.*, 2002). A recent study indicated that multiple genes regulated by the Al Resistance Transcription Factor1 (ART1) control Al-tolerance (Yamaji *et al.*, 2009). ART1 is a C2H2-type zinc-finger transcription factor and is found in the nuclei of all root cells (Yamaji *et al.*, 2009). Among the multiple genes regulated by ART1 and associated with internal and external detoxification, STAR1 and STAR2 (Huang *et al.*, 2009); *Nrat1* (Xia *et al.*, 2010); *OsFRDL4* (*OsMATE*) (Yokosho *et al.*, 2011); *OsALS1* (Huang *et al.*, 2012) and *Oryza sativa* Magnesium Transporter1 (*OsMGT1*) (Chen *et al.*, 2012) have been characterized. STAR1 and STAR2 encode for a bacterial-type ATP binding cassette (ABC) transporter complex that transports UDP–glucose (Huang *et al.*, 2009). The authors suggested that UDP-glucose (a glycoside derived compound) is released from vesicles into the apoplast by

exocytosis and that it modifies the cell walls to mask the binding sites for aluminium, resulting in aluminium tolerance in rice. Both genes are expressed mainly in the roots and are specifically induced by Al. Disruption of either genes results in increased sensitivity to Al-toxicity (Huang *et al.*, 2009).

Nrat1 belongs to the *Nramp* (natural resistance-associated macrophage protein) family and is a plasma membrane-localized transporter for trivalent Al (Xia *et al.*, 2010). *OsALS1* encodes for a half-size ABC transporter that is a member of the TAP (transporter associated with antigen processing) sub-group (Huang *et al.*, 2012). *OsALS1* is localized to the tonoplast of root cells and is responsible for sequestration of Al into vacuoles, which is required for internal detoxification of Al in rice (Huang *et al.*, 2012). *OsALS1* and *Nrat1* operate cooperatively in that Al transported by *Nrat1* is sequestered by *OsALS1* (Huang *et al.*, 2012).

OsFRDL4 that encodes for a citrate transporter and is homologous to *SbMATE* of sorghum, sharing a 70% identity at the amino acid level (Yokosho *et al.*, 2011). Knockout of this gene results in decreased citrate secretion and increased Al sensitivity. However, the contribution of the *OsFRDL4* gene in overall Al-tolerance of rice is relatively small (Yokosho *et al.*)

OsMGT1 is a plasma-membrane localized transporter for Mg in rice and its expression is specifically boosted by Al (Chen *et al.*, 2012). Up-regulation of this transporter gene is required for conferring Al tolerance by increasing Mg uptake into the cells. Knockout of *OsMGT1* resulted in increased sensitivity to Al in both solution and soil culture. It is hypothesised that Mg prevents Al binding to enzymes and other cellular components and enables its detoxification (Chen *et al.*, 2012).

1.8 Breeding for Al-tolerance in cereals

Like any other trait of economic importance, breeding method for Al-tolerance depends on the pollination biology of individual crops, the inheritance of genes controlling the trait and their gene action (Poehlman and Sleper, 1995). The first step in breeding for tolerance to Al-toxicity is acquisition of diverse genetic resources. In the Ethiopian context, the germplasm acquisition and breeding for tolerance to Al-toxicity has to follow two strategies.

For indigenous crops that have not been bred for Al-tolerance, collection of germplasm and evaluation for Al-tolerance and other economic traits is an appropriate strategy in

the short term. Studies on inheritance, gene action and follow up activities on genetic recombination and progeny evaluation will be undertaken as medium and long term goals. For globally important cereals that have been well studied, the introduction, evaluation and selection of adaptable tolerant materials can be the right entry point for the short term. Introgression of Al-tolerance gene/s from known sources into popular varieties and selection can help to develop Al-tolerant varieties with good agronomic features.

Table 1.3. Genes encoding for membrane transport protein families in different cereals

Crop	Gene family	Reference
Wheat (*Triticum aestivum*)	*TaALMT1*	(Sasaki *et al.*, 2004) (Raman *et al.*, 2005b)
	TaMATE1	(Ryan *et al.*, 2009)
Barley (*Hordeum vulgare*)	*HvMATE*	(Wang *et al.*, 2006) (Wang *et al.*, 2007)
Rye (*Secale cereale*)	*Sc ALMT1*	(Fontecha *et al.*, 2007) (Collins *et al.*, 2008)
	ScMATE	(Navas *et al.*, 2012)
Sorghum (*Sorghum bicolor*)	*SbMATE*	(Magalhaes *et al.*, 2007)
Maize (*Zea mays*)	*ZmMATE1*, *ZmMATE2*	(Maron *et al.*, 2008) (Maron *et al.*, 2010) (Maron *et al.*, 2013
	ZmNrat1	Guimaraes, *et al.*, 2014
Rice (*Oryza sativa*)	*START1* and *START2* (ABC transporters)	(Huang *et al.*, 2009)
	OsALS1 (ABC transporter member of the TAP (transporter associated with antigen processing) sub-group	(Huang *et al.*, 2012)
	Nramp (natural resistance-associated macrophage protein) family	(Xia *et al.*, 2010)
	OsFRDL4 (*OsMATE*)-Citrate transporter	(Yokosho *et al.*, 2011)
	OsMGT1-Magnesium transporter	(Chen *et al.*, 2012)

One approach to starting breeding for Al tolerant varieties is to start with germplasm collected from areas prone to acid soils. Most of Al-tolerant crop genotypes developed so far were developed from parent populations sourced from regions of the world with highly acidic soils (Rao *et al.*, 1993; Poehlman and Sleper, 1995; Ryan *et al.*, 2009). For instance among 250 bread wheat landraces originating from 21 countries, all of 25 accessions collected from highly acid soils in Nepal were found to be Al-tolerant (Stodart *et al.*, 2007). The most likely reasons for such associations are natural selection and/or human selection by early farmers (Rao *et al.*, 1993; Stodart *et al.*, 2007).

Induction of mutations using radiation or mutagenic chemicals can also be used to rapidly increase genetic variability for Al-tolerance for screening programmes. In barley and *Arabidopsis thaliana* L, mutagenic treatment with N-methyl-N-nitroso-urea (MNH) and sodium azide; and ethyl methanesulfonate (EMS), respectively, resulted in mutants with increased level of Al-tolerance, (Nawrot *et al.*, 2001; Kelly *et al.*, 2006). Acid soil/Al-tolerant variants of sorghum, rice and maize have been obtained from somaclonal variations under *in vitro* conditions (Foy *et al.*, 1993; Duncan *et al.*, 1995; Jan *et al.*, 1997; Sibov *et al.*, 1999). Genetic engineering methods have also been used for genetic and expression analysis studies and to develop genotypes with enhanced aluminium tolerance (Deborah and Tesfaye, 2003; Dharmendra *et al.*, 2011; Roy *et al.*, 2011).

1.9 Screening methods for Al-tolerance

1.9.1 Nutrient solution culture

This technique is the most common screening method for Al tolerance. It simplifies root measurement or other assay methods of Al-tolerance and allows for easy control over nutrient availability, pH, light conditions, etc. (Carver and Ownby, 1995). Regular monitoring the medium is imperative because plant root exudates can change the pH of the nutrient solution (Deborah and Tesfaye, 2003).

Magnavaca's nutrient solution for maize, sorghum and wheat, and Yoshida's nutrient solutions for rice are the most commonly used nutrient solution formulations for Al-tolerance screening (Yoshida *et al.*, 1976; Magnavaca *et al.*, 1987; Magalhaes *et al.*, 2004; Sasaki *et al.*, 2004; Magalhaes *et al.*, 2007). Nutrient solutions with low-ionic-strength and low Al concentration are usually used to mimic the ionic strength and aluminium activity found in acid soil environments (Blamey *et al.*, 1992). Accordingly, Famoso *et al.* (2010) modified Magnavaca's nutrient solution and developed an Al screening nutrient solution with a reduced ionic strength and a reduced precipitating effect on Al. This solution also increased the availability of important nutrients by reducing their interaction with Al. They called this formulation a "modified Magnavaca's nutrient solution" (Famoso *et al.*, 2010).

Different crop species vary in their sensitivity to toxic concentrations of Al. Specific Al concentrations are used to screening each cereal crop, in order to accurately discriminate between the Al-sensitivity of the available germplasm. For instance, the

concentration of Al that gives free Al^{3+} activity has been identified as 8.75 µM for wheat (Sasaki *et al.*, 2004), 27 µM for sorghum (Magalhaes *et al.*, 2007), 39 µM for maize (Pineros *et al.*, 2005) and 160 µM for rice (Famoso *et al.*, 2010).

Root tip staining and root growth measurement methods are the main methods used to measure Al-tolerance in cereals grown in a nutrient solution culture.

Root tip staining: There are several methods of root tip staining. Among these, haematoxylin staining of root tips is a widely used and powerful method (Polle *et al.*, 1978; Deborah and Tesfaye, 2003; Raman and Gustafson, 2011). Eriochrome cyanine, and lumogallion root staining methods have also been used to discriminate between tolerant and sensitive genotypes of various crop species (Junping *et al.*, 2006; Narasimhamoorthy *et al.*, 2007). These stains identify Al-sensitive genotypes by forming complexes with Al ions accumulated in the root tips of the test plants. Nitroblue tetrazolium (NBT) is another stain that has been used to identify Al-tolerant genotypes. With NBT staining, a high degree of staining has been related to high levels of Al tolerance in wheat, rye, maize, and rice (Maltais and Houde, 2002; Raman and Gustafson, 2011).

Root growth: Root growth measurements are also widely used to assess genotypes for Al-tolerance and sensitivity when tested in nutrient solutions (Baier *et al.*, 1995; Carver and Ownby, 1995). The root growth method considers two Al tolerance parameters: Root growth (RG) and root tolerance index (RTI) (Baier *et al.*, 1995). The RG parameter measures root growth under Al stress. Genotypes that exhibit longer roots and greater root densities under toxic concentration of Al are tolerant to Al. RTI or Relative Root Growth (RRG) is computed as the ratio of root growth under Al stress to root growth without Al stress (Hede *et al.*, 2002; Raman and Gustafson, 2011). Plants with higher RTI values are more tolerant of Al. RTI needs an experimental set up that allows for the measurement of root growth parameters under both Al stress and without Al stress. Al tolerance can be a combination of two genes/ alleles controlling inherent root vigour, and root tolerance to Al. RTI removes the effect of genes controlling root vigour by taking relative growth of the genotype in an Al solution and comparing it with its growth without Al. The one drawback of RTI as a measurement is that environmentally sensitive genotypes that grow slowly under non-

stressed conditions can have high RTI values and may appear Al-tolerant (DallAgnol *et al.*, 1996; Hede *et al.*, 2002).

When the two assessment methods are compared, root staining evaluates the accumulation of aluminium in the roots, disregarding the possibility of an accumulation of aluminium in other plant parts. Hence, it may misclassify plants when there is genetic difference between plants in their mechanisms of Al tolerance. Root growth methods, however, avoids this complexity because they measure the genetic potential of plants to overcome the known effect of root growth inhibition (Raman and Gustafson, 2011).

The shortcomings of root measurement methods includes the fact that they are time consuming. They are also dependent on non-genetic attributes of seeds such age, seed size, and physiological conditions which can lead to erroneous inferences (Raman and Gustafson, 2011). Nevertheless, these drawbacks can be overcome by using seeds with similar physical and physiological conditions (Baier *et al.*, 1995). It is also important to germinate seeds and select for uniform seedlings before evaluating for Al tolerance (Hede *et al.*, 2002).

1.9.2 *In vitro* (tissue culture) screening method

Compared to screening under field condition, the *in vitro* technique is relatively fast and can be done at an early stage of plant development. This method involves the evaluation of callus growth grown on an acid tissue culture medium containing a toxic concentration of aluminium, compared with an aluminium free acidic medium (Conner and Meredith, 1985; Deborah and Tesfaye, 2003; Dharmendra *et al.*, 2011). The underlying assumption for the development of tolerant materials from callus culture is that tolerance at a cell culture level will operates in whole-plants under field conditions. This technique has been used to identify Al-tolerant plants. However, its economic feasibility is questionable for some species, and is challenging for many cereals that are not easily grown in tissue culture (DallAgnol *et al.*, 1996).

There are also technical challenges with *in vitro* screening. In order to emulate problems of acid soils with Al-toxicity problem, several modifications have to be made (Conner and Meredith, 1985). For instance, the pH has to be reduced to about 4.0. However, at pH of 4.0, agar does not solidify when autoclaved. In order to overcome this, a high concentration of Gelrite (up to 14 g. L^{-1}) (Jan *et al.*, 1997) and 5-9 g.L^{-1} is

used (Ramgareeb *et al.*, 1999). Secondly, aluminium forms precipitates with various nutrients in the medium, so it is difficult to control the availability and activity of the toxic aluminium species in the medium (Ramgareeb *et al.*, 1999). Hence, the use of a chemical equilibrium speciation model has been suggested to predict the availability and activity of the toxic Al species (Ramgareeb *et al.*, 1999; Shaff *et al.*, 2010).

1.9.3 Soil based screening

Screening crops in acid soils usually follows preliminary screenings made in solution culture. It is preferable to conduct soil-based screening in soils taken from the target production area or to represent the target production area (Carver and Ownby, 1995). Root growth and root tolerance index assessment methods discussed above under nutrient solution culture are commonly used. To compute a tolerance index, plants are grown in an acid soil limed to a non-toxic level, and in unlimed soil. Relative root dry-matter and shoot dry-matter are used to evaluate the materials because the Relative value = the value with aluminium stress/value without aluminium stress (Foy *et al.*, 1987; Hill *et al.*, 1989; Foy and Murray, 1998; Liu, 2005). The advantage of using soil based screening methods compared to nutrient solution culture is that they take into consideration other soil factors that may influence Al tolerance (Ring *et al.*, 1993).

1.9.4 Field evaluation

The reason for breeding for Al-tolerant crop varieties is to make target areas with acid soils more productive. Hence, evaluation of selected varieties for yield and other economically important traits at target production areas is imperative. Field evaluation is usually conducted in pairs of lime amended and naturally acidic plots for all the genotypes to be evaluated, and tolerance indices are computed for analysis (Carver and Ownby, 1995; Johnson *et al.*, 1997). In field evaluation, to the major challenge is to avoid heterogeneity effects in the soil, associated with non- treatment factors such as soil variability, differential soil compaction and the effect of soil-borne pathogens with a patchy distribution.

1.10 Molecular marker assisted breeding for Al-tolerance in cereals

Devos and Gale (2000) found extensive synteny or colinearity among the genomes of rice, wheat, barley, rye, oat, maize and sorghum in a comparative mapping study. This opens up the possibility of screening for Al-tolerance loci in cereals using a set of common DNA markers linked to Al-tolerance (Raman and Gustafson, 2011).

Currently, diagnostic markers associated with the candidate genes *TaALMT*, *Sc ALMT1*, *HvMATE, SbMATE* and *ZmMATE1* have been developed (Table 1.2). These genes are mainly correlated with Al-tolerance in wheat, barley and sorghum, respectively. Molecular markers for Al-tolerance have been applied in breeding programmes to monitor for the presence of the desired alleles in different genetic background and in genetic diversity studies (Raman and Gustafson, 2011).

1.11 Conclusion

Much of the arable land in Ethiopia is negatively affected by soil acidity and Al-toxicity. These areas are mainly found in the high rainfall areas of the north western, western, south western and southern parts of the country. Use of lime in the areas affected by acid soils is affected by its local unavailability, its high cost, and the difficulties associated with its transport, and the application of the appropriate tonnages of lime often recommended to offset the of the buffering capacity of the acid soils. On the other hand, competing uses of crop residues for fuel, animal feed and construction material hinders the widespread use of compost and animal manure for soil acidity management. In contrast, Al-tolerant crop varieties can be used as the primary component of an integrated acid soil management strategy for Ethiopia. However, this will need a shift in research priorities to enable the start of active breeding of staple crops for adaptation to the acid soils of Ethiopia, and specifically to tef breeding.

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CHAPTER 2

Soil acidity: Importance, assessment of perceived causes and indicators, coping strategies and implications in cereal based mixed-farming system of north western Ethiopia

Abstract

Soil acidity is one of major crop production constraints in north western Ethiopia. Nonetheless, information on the extent of soil acidity across land-uses is scarce. A participatory rural appraisal (PRA) was conducted in three Districts of north western Ethiopia affected by acid soils in order to: 1) Assess the state of soil acidity under multiple land-uses; 2) Determine the perceived causes and indicators of soil acidity, and coping strategies of farmers dealing with soil acidity; and 3) Assess the importance of soil acidity as a crop production constraint. Semi- structured interviews, group discussions and soil analyses were the main techniques used to generate data. Soil samples were collected from five dominant land-uses and were analysed for soil pH, exchangeable acidity and other physico-chemical properties. Farmers' perceptions were that the causes of soil acidity included: Soil erosion; competing use of local resources and poor nutrient recycling; the abandoning traditional fertility management practices; and the minimal and unbalanced use of external inputs. The farmers indirectly implicated the exclusive use of acid-forming inorganic fertilizers to exhaustion of the soil. Soil erosion, soil acidity, the high cost of mineral fertilizers and lime, cash shortages, and the unavailability of seeds of adapted varieties were viewed as the top ranking constraints. Species tolerance to soil acidity was found to be one of the major factors that influenced crop choice by farmers. Various land and soil characteristics, plant growth attributes, changes in genetic diversity were mentioned as indicators of soil acidity. The physico-chemical properties of the soils showed variation across land-uses and study sites. Nonetheless, the $pH_{(H2O)}$ of most of the soils in the study sites were in a strongly acidic range (4.6–5.5). *Gashena Akayita* of *Banja* District was the most acidic of all with high levels of exchangeable Al. At all the study sites, exchangeable Al was detected in soils having a pH of less than 5.0. Among the land-uses, eucalyptus fields were the most acidic followed by crop outfields and grazing lands, in that order. Mn toxicity was found to be a potential problem for the Districts of *Enguti* and *Enerata*. Farmers' perceptions of soil acidity were in agreement

with the soil test results. The limitations of the current coping strategies and the need to avail compatible technologies that ensure sustainable soil management are discussed.

Key words: Farmers' perceptions**,** soil acidity, coping strategy

2.1 Introduction

Land degradation is an important global problem affecting current and future food production and rural livelihoods (Scherr and Yadav, 1996; Bruinsma, 2009; Godfray *et al.*, 2010; Eswaran *et al.*, 2001). The growing decline in productivity of soils due to soil acidity is one aspect of land degradation constraining crop production worldwide. Acid soils constitute 40% of the world's total ice-free land. In Africa, 22% of the land, or 659 million ha of land have soil acidity problems (von Uexk¨ull and Mutert, 1995).

In Ethiopia, acid soils with a pH of below 5.5 in the surface layer constitute about 13.2% of the total land area (Schlede, 1989) and are mainly distributed in the western, north western, south western and southern parts of the country (Schlede, 1989; Abebe, 2007). The importance of the areas affected by acid soils lies in the fact that they are found in the high rainfall areas with good agricultural potential. In contrast, the eastern regions of Ethiopia have neutral soils, but suffer from recurrent droughts (Abebe, 2007; IFPRI, 2010).

The promotion of mineral fertilizers, compost and lime use, along with soil and water conservation practices, have been the main strategies promoted by the government extension service to counter the problem of soil acidity. Nonetheless, variability in agro-ecologies, local resource endowment and the limited capacity of small-scale farmers to invest in such options have limited their impact in the management of acid soils (Alemneh, 2003).

Land-use is one of the anthropogenic factor that affect pH and other physico-chemical properties (Behera and Shukla, 2015). However, most of the strategies under promotion are focused on the reclamation of crop fields, with no or little consideration given to the varied land-uses prevailing in the existing mixed farming systems.

Farmers are a repository of indigenous knowledge, and have practical experience on how to adapt their farming practices to changing socioeconomic and biophysical

circumstances (Freudenberger, 1994; Dixon *et al.*, 2001). Hence, farmers' participation in the process of problem identification and technology development may help to identify management strategies that are compatible with local socio-economic and biophysical environments (Freudenberger, 1994; Nabhan, 1999; Dixon *et al.*, 2001). Furthermore, participatory approaches that involve local stakeholders help to avoid the inadequacies associated with quantitative methods that often are expert biased (Vaidya and Mayer, 2014). Various studies also shown a correlation between farmers' knowledge and scientific evidence on the causes and indicators of land degradation related to soil fertility decline (Malley *et al.*, 2006; Karltun *et al.*, 2013; Vaidya and Mayer, 2014). Alemneh (2003) noted that the lack of participatory approaches has undermined soil fertility initiatives that could have improved soil productivity and intensification of the agriculture by combining high and low input technologies suitable for the small-scale farmers of Ethiopia. Research endeavours specifically targeting acid soil environments are in their infancy and are constrained by a paucity of information. This research was carried out in order to assess the state of soil acidity across multiple land-uses and to document farmers' knowledge and understanding of soil acidity. The information generated from this study of several agro-ecologies affected by acid soils is expected to help in designing appropriate interventions for the study areas and similar environments.

2.2 Material and methods

2.2.1 Description of the study sites

The study was conducted at the *Enguti*, *Gashena Akayita* and *Enerata* peasant associations (PA[2]s) in the *Mecha*, *Banja* and *Gozamin* Districts of West Gojjam, Awi and East Gojjam administrative zones in north western Ethiopia, respectively, from December 2012 to January 2013 (Figure 2.1). The study areas were carefully selected to represent areas severely affected by acid soils, and where the farmers are involved in mixed farming systems.

According to the traditional agroecological classification, which is mainly based on altitude, the *Dega* and *Woinadega* zones include areas with elevations of 2300-3200m and 1500-2300 m above sea level, respectively (IFPRI and CSA, 2006). *Gashena*

[2] *PA is the smallest administrative unit in the government structure.

Akayita (2500-2700 m) and *Enerata* (2400-2700 m) fall into the *Dega* zone, whereas *Enguti* (1900-2100m) falls into the *Woinadega* Zone. In terms of their temperature and moisture regimes, *Gashena Akayita* is cool and sub-humid, whereas *Enerata* is cool and moist. *Enguti* is tepid and moist, allowing for the production of a greater diversity of crop species than the other two sites. The rainfall pattern is unimodal across all the study sites. *Gashena Akayita* usually receives the highest rainfall (Figure 2.2).

The mean size of landholding per household was 0.5ha for *Banja,* and 1.5ha for the *Mecha* and *Gozamin* Districts. The soil class of *Gashena Akayita* is predominantly Acrisol (Ultisol), whereas those of *Enerata* and *Enguti* are mostly Nitosols (Yihenew, 2002; IFPRI and CSA, 2006).

2.2.2 Data collection

Data collection involved secondary data gathering from local sources, participatory rural appraisal (PRA) techniques, and a comprehensive soil sampling programme.

Secondary data collection: Included secondary data gathering on soil type, land-use, vegetation cover, major crops produced, animal and human population, etc.

Direct Observation: A transect-walk was made at each site in the company of key informants in order to better understand the farming systems in terms of land-use, land form, vegetation cover, etc. through visual observation, and informal discussions with the people of the area.

A semi-structured interview: A semi-structured interview was administered to 61 farmers, i.e., 20 farmers at *Enguti* and *Enerata,* and 21 at *Gashena Akayita,* using a checklist of topics and guide questions. The farmers were randomly selected and the number of interviewees was determined based on the extent of data saturation achieved (Mason, 2010). Data were collected on farmers' perceptions of the major production constraints affecting tef, their current coping strategies and their limitations; and the factors influencing their choice of major crops and varieties being grown. A key informant interview was also administered to five agricultural development workers at each site on the extent of soil acidity and farmers' coping strategies. Iteration and probing techniques were used to generate the information adequately and precisely, with a degree of cross-checking.

Group discussion: Group discussions involving 15 farmers at each of *Enguti* and *Gashena Akayita* and 30 farmers at *Enerata* were held. Ranking of major production constraints that had been identified through the semi-structured interview, and identification of the prevalent soil fertility management methods being used were carried out through group discussions. Subsequently, farmers' perceived indicators and causes of soil acidity; the spatial distribution of acid soils; and changing patterns in land-use were assessed using cause-and-effect analysis, trend analysis, listing and sorting, and the spectrum of PRA tools. The initial outcomes were then confirmed through discussions leading to consensus among the participants.

Soil sample collection: A soil sampling strategy across the different land-uses was established based on secondary data analysis, and the outcomes of the transect-walks and the group discussions. Accordingly, the land-use and the management types were categorized into six classes: Homestead plots, limed outfields, unlimed outfields, eucalyptus fields, natural forest, and grazing land. Soil samples were collected from each land-use type except for the natural forest at *Enerata* and the limed outfields from *Enguti*. A field or a plot with an area of about 2ha was taken as a sampling unit (Motsara and Roy, 2008). Each composite soil sample was generated by samples from a minimum of 5 fields scattered over the PAs for the crop and eucalyptus fields. For natural forest and grazing land, all available patches were sampled. From each sampling unit (2ha area), a minimum of 5 samples were collected in a diagonal sampling pattern, using a 20cm auger. Overall, sixteen composite samples were collected to represent the different land-uses and crop management systems across the three study areas. Soil samples were air dried, crushed and sieved through a 2 mm diameter mesh, combined and thoroughly mixed for each land-use and study area. A quartering method was used to extract the final 1 kg of soil needed for analysis.

Soil analysis: Soil samples were analysed in the laboratory of the Amhara Design and Supervision Works Enterprise, Soil Chemistry and Water Quality Section, Bahir Dar, Ethiopia. FAO laboratory procedures as outlined in Motsara and Roy (2008) were used to conduct the analysis of the specific physico-chemical properties. The pH was potentiometrically measured in the supernatant suspension of a 1:2.5 soil: H_2O/ KCl mixture. The particle size was determined using a hydrometer method (Bouyoucos, 1951).The pH (H_20) was measured in 1:2.5 soil to water and soil. CEC was determined

by extracting with 1N ammonium acetate at pH 7.0 and using a titration method. Exchangeable bases were measured by an atomic absorption spectrophotometer (AAS). Mn was extracted by DTPA (diethylenetriamine penta acetic acid) and determined by AAS (Lindsay and Norvell, 1978). Exchangeable acidity and exchangeable aluminium were determined by titration methods after leaching with a neutral 1N KCl solution. Organic carbon content was determined using Walkley and Black (1934) titration method. The available phosphorus content was determined by the Olsen method (Olsen *et al.*, 1954).The total nitrogen was analysed by the semi-micro Kjeldahl procedure of Hitchcock and Belden (1933).

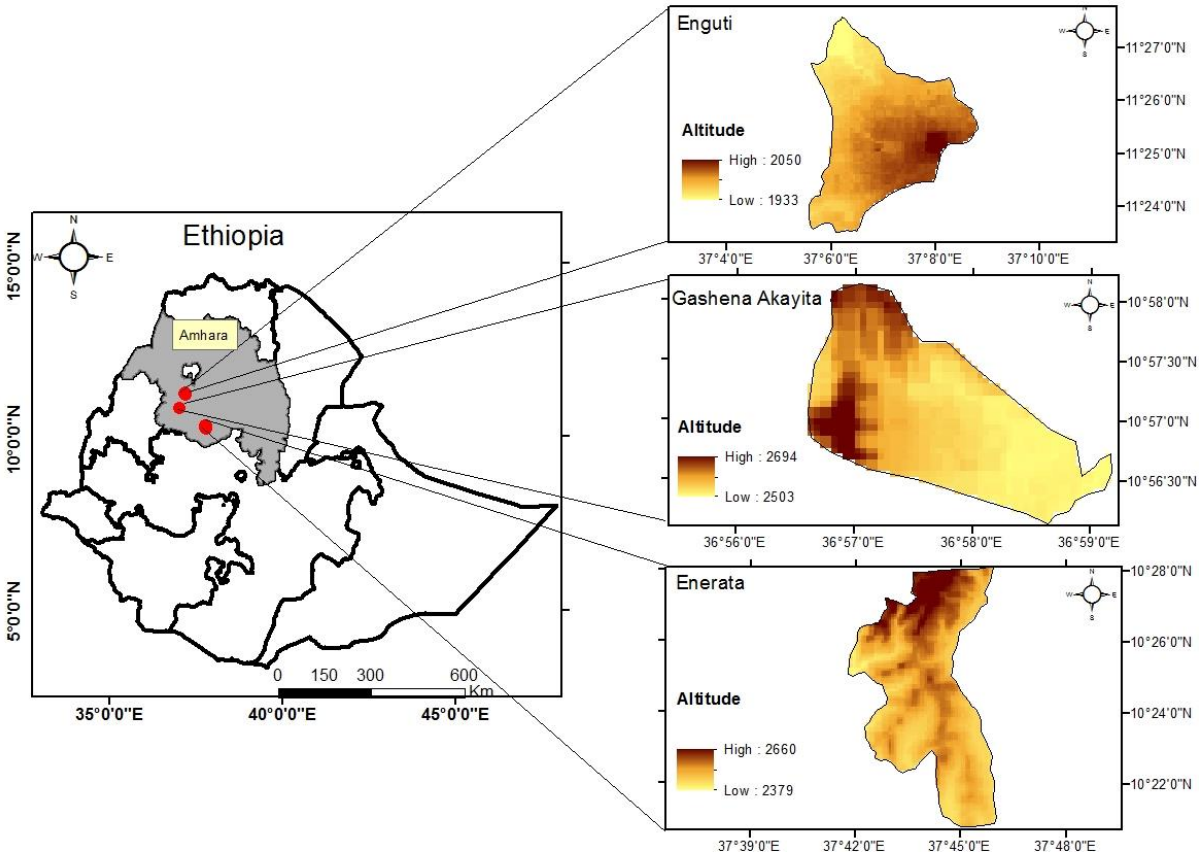

Figure 2.1. Geographical location and altitudinal range of the study areas

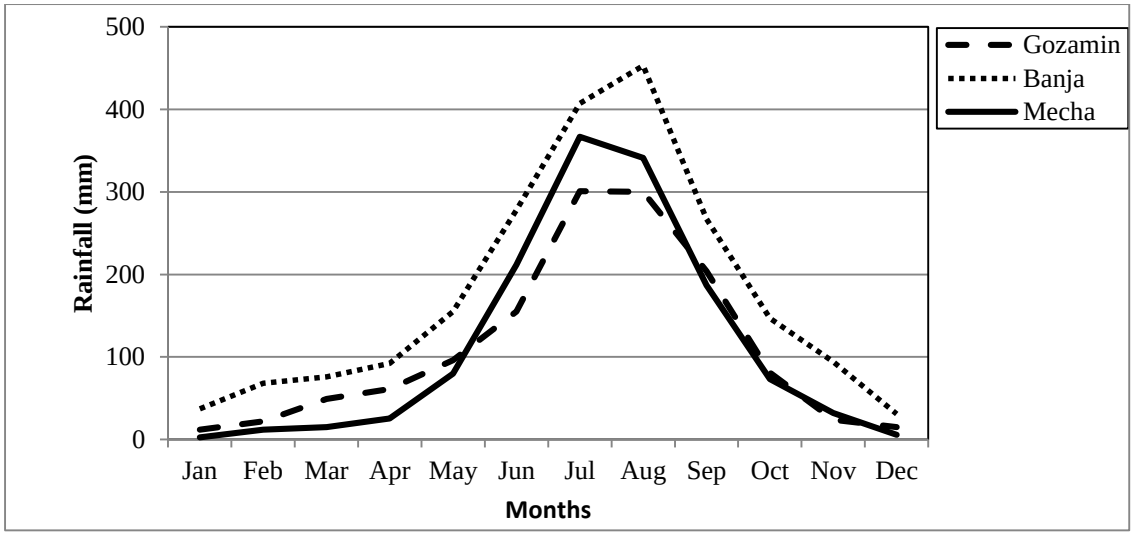

Figure 2.2. Rainfall pattern of the study areas: Mean of 10 years interpolated by an FAO local climate estimator (FAO, 2005)

2.3 Results

2.3.1 Predominant land-uses of the farming system

Sedentary mixed crop-livestock farming characterized the farming system in all the study areas. The proportion of land allocated to different agricultural uses ranged from 63% in *Mecha* to 93% in *Banja* (Table 2.1). *Gozamin* and *Banja* host zonal towns and consequently had a smaller rural population. *Banja* was the most densely populated and had the smallest mean land holding per household. Among the agricultural land-uses, crop production was first in terms of area of usage, followed by grazing land. Tenure of grazing lands is predominantly communal in all the study areas. A larger percentage of total land area was allocated for grazing at *Gozamin*. Nonetheless, most of the communal grazing lands were overgrazed and severely eroded. Substantial areas of the grazing lands were occupied by weed species with no feed value. The farmers collected cattle dung dropped on grazing lands for fuel during the dry season with no restrictions on this practice. Due to the low productivity of grazing lands and the high livestock populations, farmers rely on crop residues to feed their livestock. Most farmers at *Banja* used the most acidic outfields for pastures in order to cope with the problem of soil acidity. However, efforts to improve the productivity of either the communal or the private grazing lands were negligible.

A strong link between crop production and livestock rearing was demonstrated in the study areas. Horses and oxen were used in the extreme highland parts and mid-altitude areas, respectively, to provide draught power for land preparation. Threshing and transport of crops was also carried out by livestock. Horses and donkeys were the predominant livestock used to transport agricultural produce to the markets, and bring in important inputs such as fertilizers and lime to the farms. Livestock also provided manure to replenish soil fertility, principally in homestead areas. In the *Banja* area, *hura* or night corralling of cattle on outfields is longstanding popular practice used to replenish the fertility through direct application of manure and urine.

In the *Mecha* and *Gozamin* areas, the share of natural forest was negligible and restricted to the area of land surroundings ancient churches and monasteries. Despite having the least mean land holding per household, encroachment of natural forest seems minimal in the *Banja* area. Consequently, the proportion of land covered by natural forest in this District was significant. Considerable rural household income was derived from the sale of bamboo (*Arundinaria spp.* Michx.*)* and eucalyptus products in the study areas. Plantations of green wattle *(Acacia decurrens* (Wendl.) Willd.) are rapidly expanding onto acidic soils that no longer support good crop growth. Private eucalyptus plots constitute the primary plantation forest cover in *Gozamin* and *Mecha*. Soil acidity and the prohibitive cost of fertilizer were among the main driving factors forcing farmers to switch to eucalyptus farming. Traditional agroforestry that involves the deliberate cultivation of nitrogen fixing forestry species such as *Croton macrostychus* Hochst. ex Ferret et Galinier and *Cordia spp.* L. also contributed to the vegetation cover in the *Mecha* area.

Table 2.1. Selected indicators of farming system, landuse characteristics, and climatic features of the study districts

	Districts		
Variables	***Gozamin***	***Banja***	***Mecha***
Total Population*	225,638	126,546	323,374
Rural Population (%)*	63	77	91
Population density (persons/km^2)	182.0	249.1	218.3
Mean land holding (ha)	<1.5	<0.5	<1.5
Total area for all land-uses (ha)	121,781	30,217	156,027
Soil and Climatic features			
Dominant soil type	Nitisol	Acrisol	Nitisol
Altitude (m) (only the study sites)	2400-2700	2500-2700	1900-2100
Annual rainfall (mm)*	1200-1300	2100-2200	1300-1400
Mean Min/ Max Temperature (^{o}C)*	10/22	8.5/24	12/27
Major agricultural land-uses (%)	76.4	93	63
Cultivated area (ha) (%)	44,488 (36.5)	12,277 (40.6)	72,138 (46.2)
Grazing land (ha) (%)	32,936 (27)	3443 (11.4)	15,591 (10)
Natural forest (ha) (%)	15,594(12.8)**	2679 (8.9)	5971 (3.8)
Plantation forest (ha) (%)		9694.5 (32.1)	3106 (1.99)
Livestock population			
Cattle	129,158	73,820	300,890
Shoats	121,998	102,416	175,619
Equines	21,838	26,622	35,355

Source : Respective zonal and district offices of agriculture
*2012 National Statistics (Abstract), CSA 2013; ** total for both plantation and natural forest.

2.3.2 Major crops grown

The percentage of land area allotted for crop production varied across the study sites. The largest share (46%) was allotted for crop production in *Mecha* and the least was in *Gozamin* (36.5%). Crop production was principally cereal based. At *Banja,* which is the most acidic environment, the main crops were tef (*Eragrostis tef* Zucc. Trotter) and Irish potato (*Solanum tuberosum* L.) grown on over 50% of the cultivated land (Table 2.2). Triticale (*X Triticosecale* Wittmark), a newly introduced crop to the country, occupied over 14% of the cultivated land in *Banja* (Table 2.2).

Farmers selected specific crops and varieties to grow, based on various determining factors (Table 2.3). Adaptability to acid soils was among the major determining factors in crop selection. Brown seeded tef landraces called *Dabo* were widely grown in *Gashena Akayita* and *Enerata*. Compared to white seeded tef varieties, these landraces fetched lower market prices. However, farmers valued the better

adaptability of the brown seeded tef landraces to their acidic soils and the cooler temperatures. These landraces were grown on acidic outfields with a minimum of fertilizer applications. The dominance of brown seeded tef landraces in the highland ecologies of the country has been reported previously (NRC, 1996).

Potato was the only non-cereal crop produced on substantial scale in all the study sites. In addition to other factors, its natural adaptation to acidic soils and to cool climates (Jadhave and Kadam, 1998) explain its widespread production in the study areas.

Triticale was introduced to the farming system of *Banja*, *Gozamin* and similar acid soil prone areas a few years ago. In addition to threshing difficulties, farmers were not accustomed to its utilization. Nevertheless, its cultivation has been rapidly expanding in the farming systems owing to its adaptability to acidic outfields. Triticale is classified as an acid tolerant crop owing to the genes for tolerance to Al-toxicity that it has derived from the rye genome (Niedziela *et al.*, 2012).

White lupin (*Lupinus albus* L.) and oat (*Avena sativa* L.*)* were also produced on substantial areas, mainly on outfields. White lupin can fix nitrogen on a low pH and in P deficient, acidic soils (Vance, 2001), and can tolerate Al-toxicity in acid soils (Wang *et al.*, 2007). Oat is also considered to be naturally Al-tolerant and is commonly grown in rotation with crops such as potato (Foy *et al.*, 1987; Radmer *et al.*, 2012). In addition to Al-tolerance, oat has been reported to be tolerant to Mn toxicity (Sillanpaa, 1972).

Finger millet *(Eleusine corocana* (L.) Gaertn.*)* occupied over 22% of total area cultivated in *Mecha*. The farmers reported that it is highly adapted to the acidic outfields and demand less mineral fertilizer than maize. The reaction of finger millet to soil acidity and Al-toxicity is not known. However, its capacity to mobilize rock phosphate has been reported to be stronger than most cereals (Flack *et al.*, 1987).

Generally, there was a trend to commit more land to growing acid tolerant species and landraces, suggesting that soil acidity has become one of the determining factors for selection of crops.

Table 2.2. The top five crops grown in the study areas during 2012 cropping season

District	Crop	Area(ha)	% total area
Banaja (Gashena Akayita)	Total area	12227	
	Tef	3323	27.2
	Potato	3012	24.6
	Triticale	1764	14.4
	Wheat	1152	9.4
	Maize	900	7.4
Gozamin (Enerata)	Total area	44417	
	Wheat	13903	31.3
	Tef	10131	22.8
	Maize	5079	11.4
	Barley	3050	6.9
	Potato	2489	5.6
Mecha (Enguti)	Total area	69676.35	
	Maize	29732	42.67
	F.millet	15349	22.03
	Tef	6851	9.83
	Noug	3052	4.38
	Potato	2680	3.85

Source: Respective District Offices of Agricultural Development

Table 2.3. Major crops grown and factors affecting their choice assessed by semi-structured interview of farmers (n=61)

Study sites	Crops	Determinant factors										
		High yield	House Hold food security	Crop residue for feed	Market value	Crop rotation	Early maturity	Demands less fertilizer	Tolerates/ adapted to soil acidity	Other values of residue	Tolerance to biotic stresses	Fits to double cropping
Banaja (*G. Akayita*) (n=20)	Tef		*	*	*		*	*	*	*	*	*
	Potato	*	*		*	*	*	*	*			*
	Triticale	*	*	*				*	*	*		
	Wheat	*			*					*		
	Maize	*	*	*	*					*		
Gozamin (*Enerata*) (n=21)	Wheat	*			*					*		
	Tef		*	*	*		*	*	*	*	*	
	Maize	*	*	*	*					*		
	Barley		*	*	*		*	*			*	
	Potato	*	*		*	*	*		*			
Mecha (*Enguti*) (n=20)	Maize	*	*	*	*					*		
	F.millet		*	*	*	*		*	*	*	*	
	Tef		*	*	*		*	*	*	*	*	
	Noug			*	*	*		*	*		*	
	Potato	*	*		*	*						

2.3.3 Farmers' perceptions and physico-chemical properties of the soils

The textural class of soils from *Enerata* and *Enguti* was clay whereas that of *Akayita* was mainly loamy. Variations were observed in the physical properties of soils across different land-uses. Outfields of *Enerata* belonged to the heavy clay class and had very low organic carbon (OC) levels (<2%), while homestead, grazing land and eucalyptus plots had a clay texture and higher OC levels. Generally, soil samples from homesteads and grazing land had relatively high OC contents (Table 2.4). Homestead soils benefited from organic matter applied in the form of household refuse and animal manure. The absence of tillage practices and the slow decomposition of manure might contribute to the relatively high OC levels of the grazing lands in the study areas.

Table 2.4. Soil texture components, and levels of organic carbon and organic matter of soil samples from three Districts and with multiple land-uses

Site	Land-use	Texture (%)			Class	OC (%)	OMC (%)
		sand	clay	silt			
Enerata	Limed	13	65	22	heavy clay	0.94	1.61
	Unlimed	11	67	22	heavy clay	0.82	1.41
	Homestead	19	53	28	clay	2.22	3.83
	Grazing land	21	49	30	clay	2.11	3.63
	Eucalyptus	14	59	27	clay	1.95	3.36
Akayita	Limed	23	21	56	Silt loam	2.96	5.11
	Unlimed	13	21	66	Silt loam	3	5.18
	Homestead	61	11	28	Sandy loam	3.96	5.11
	Grazing land	35	21	44	Loam	3.61	6.22
	Eucalyptus	35	23	42	Loam	0.7	1.21
	Nat. forest	47	9	44	Loam	1.76	3.03
Enguti	Unlimed	5	69	26	heavy clay	2.57	4.44
	Homestead	19	53	28	clay	2.94	5.08
	Grazing land	23	47	30	clay	4.25	7.33
	Eucalyptus	9	65	26	heavy clay	2.38	4.1
	Nat. forest	23	47	30	clay	1.48	2.55

In his study of Ethiopian soils, Landon (1991) found the carbon content of nearly all the study sites and land-uses to fall in the very low (<2%) to low (2-4%) range (Table 2.4). Soils of *Gashena Akayita* had the highest OC levels for all land-use types except for eucalyptus. The farmers reported that they heavily rely on crop residue for animal feed. Dried animal manure is also widely used as a fuel for cooking and heating. Hence, the low OC content of the soils can be partly attributed to the ongoing low

return of organic matter and crop residues to the soil. The extremely low OC levels of the limed and unlimed crop lands may be associated with the many tillage events required to create a fine seed bed for the tiny seeds of tef. Hence tef cultivation probably exacerbates the loss of OC through oxidation resulting from tillage.

According to the FAO classification of soils outlined by Motsara and Roy (2008), the $pH_{(H2O)}$ of most of the soils in the study sites were in the strongly acidic range (4.6–5.5) (Table 2.5). Only, one soil sample taken from homesteads at *Enguti* had a moderately acidic $pH_{(H2O)}$ of (5.6–6.5). A sample from the outfields of *Gashena Akayita* belonged to the extremely acid class (<4.6). Among the study sites, *Gashena Akayita* was the most acidic environment, followed by *Enguti* and *Enerata*. Except for samples from natural forest and homesteads, the $pH_{(H2O)}$ values of all samples from *Gashena Akayita* were below 5.0. Among land-uses, samples from unlimed outfields and eucalyptus plots were the most acidic (Table 2.5).

At all the study sites, exchangeable Al was detected in all the soils having a pH of less than 5.0 (Table 2.5). At *Enerata,* only the soil samples collected from eucalyptus fields were positive for exchangeable Al (1 cmol Kg^{-1} of soil). At *Enguti,* the levels of exchangeable Al were 0.56 and 0.24 cmol kg^{-1} of soil for crop outfields and eucalyptus plots, respectively. At *Gashena Akayita* exchangeable Al was detected under all the land-uses, except in samples from natural forest. Since the soil samples were composites, the detected Al levels were mean values. Al-toxicity was probably a problem in outfields of all the study sites, and those of *Gashena Akayita* in particular (Table 2.5). High levels of soil acidity, with appreciable level of exchangeable Al, were previously reported for sites in the *Mecha* and *Banja* areas (Yihenew, 2002).

Interpretation of available phosphorous varies depending on the crop demand (Sanchez, 2007). Using Olsen's method, an available P content of less than 4.0 mg kg^{-1} of soil is deficient, and above 8.0 mg kg^{-1} of soil is adequate for cereals. For potato, an available P content of less than 11 mg kg^{-1} of soil is deficient, and above 21mg kg^{-1} is adequate (Cooke, 1967). Accordingly, except for homestead soils, the Nitosols of *Enguti* and *Enerata* are less than, or close to, the deficiency threshold. The soil class of these two sites was predominantly Nitosols and such soils are inherently strongly P fixing soils (Driessen *et al.*, 2001). Yihenew (2002) also reported q low P content for areas with Nitisol soils in north western Ethiopia. The highest levels of available P were obtained from homestead samples for all the study sites. At *Gashena Akayita* the P

content of soils from all the land-uses except that of natural forest was above 8 mg.kg^{-1}. At *Gashena Akayita* the available P content of the outfields and the homesteads was above 8mg kg-1. High levels of available P were previously reported for the soils of *Banja* District (*Gashena Akayita*) (Yihenew, 2002).

For total N content, as determined by the Kjeldahl method, an N level of less than 0.1% is very low; 0.1-0.2% is low; 0.2-0.5 medium; 0.5-1 high; and above 1% is very high (Landon, 1991). Accordingly, the N content of the soils from *Enerata*, *Enguti* and *Gashena Akayita* were predominantly in very low, low and medium ranges, respectively. Overall, the soil samples taken from homestead, natural forest and grazing lands had relatively high N levels (Table 2.5).

As per Landon's (1991) classification, the CEC of the soils samples from all the study sites and the land-uses fell into the categories of high (25-40 cmol kg^{-1} of soil) and very high (>40 cmol kg^{-1} of soil). The base saturation of the soils of the study sites fell into the medium (20-60%) and high (>60%) ranges (Landon, 1991). Despite their clay and heavy clay texture, the CEC values of soils from the *Enerata* and *Enguti* were lower than that of *Gashena Akayita*. This can be attributed to the relatively high organic matter content of soils from *Gashena Akayita*. Given that both Nitisols and Acrisols predominantly contain 1:1 kaolinitic clay particles (Driessen *et al.*, 2001), the difference cannot be associated with the nature of the clay minerals.

Of all the study sites, the soils from *Gashena Akayita* had the lowest level of base saturation. All the soil samples collected from all land-uses at *Enerata* had higher base saturation levels than those from *Enguti*. Among all the land-uses, crop outfields and eucalyptus plots had the lowest base saturation. Even though the overall base saturation status is one of the indicators of soil fertility status, the relative balance among the bases is a more important indicator of soil fertility, and availability of nutrients to plants (Landon, 1991).

Exchangeable Ca values of above 10.0 cmol kg^{-1} soils are considered to be high and values less than 4 are low (Landon, 1991). Except for the crop outfields of *Gashena Akayita* (1.56 cmol kg^{-1} of soil), all the study sites and land-uses had calcium levels of greater than 4.0 cmol kg^{-1} of soil. Nevertheless, soil pH, CEC and proportion of other cations such as Mg and K affect availability and uptake of calcium by plants (Landon, 1991).

In the tropics, Mg deficiency occurs when the exchangeable Mg falls below 0.5 cmol $kg^{-1}$of soil, whereas Mg levels of above 4.0 cmol kg^{-1} of soil are considered to be high (Landon, 1991). However, the availability of Mg is affected by the levels of other cations such as Ca and K. The availability of magnesium decreases when the ratio of Ca:Mg is above 5:1. Conversely, when the proportion of Mg is higher than that of Ca, then the availability of Ca to the plant will be affected negatively (Landon, 1991). In this study, all the sites and the land-uses had Mg levels of above 0.5 cmol kg^{-1} soil. For most of the land-uses at *Enerata* and *Enguti*, the Ca:Mg ratio was close to or less than the desired ratio of 5:1. At *Gashena Akayita* the Ca:Mg ratio for crop outfields and eucalyptus plots was extremely low, which reflected a low level of available Ca, and the need to apply Ca in the form of calcitic lime to improve both the soil pH and to provide Ca for plant nutrition. High levels of exchangeable K also affect the uptake of Mg by plants. Potassium to magnesium ratios of above 2:1 are likely to supress the uptake of Mg by plants, especially in low Mg soils (MAFF, 1967). However, in this study, except for homestead soils of *Gashena Akayita*, all the study sites and the land-uses had K:Mg ratios of less than 2:1. Furthermore, as Mg levels of all the samples were above 0.5 cmol kg^{-1} of soil, so Mg uptake problem for these soils would be unlikely (Table 2.5).

Responses to K fertilizer application are unlikely when the exchangeable K content of the soil is above 0.4 cmol kg^{-1} soil (Landon, 1991). Nevertheless, the availability and uptake of K is affected by its balance with other cations. Hence, an exchangeable potassium percentage (EPP) (exchangeable potassium expressed as percentage of total CEC) of 2% is recommended as the minimum EPP level to avoid K deficiency in humid, tropical soils (Boyer, 1972). In this study, the exchangeable K content of the soil samples from all the study sites and land-uses, including crop outfields was above 0.4 cmol kg^{-1} of soil. However, the EPP of the study sites and the different land-uses showed considerable variation. Crop outfields had EPP values of less than 2% across all the study sites, reflecting the need to apply K. The highest EPP was recorded for homestead soils across all the sites. Exchangeable Na content of all the soil samples was below threshold levels of potentially sodic soils, i.e., >1.0 cmol kg^{-1} of soil (Landon, 1991). ECe values (<2.0) for all the surveyed sites also indicated that salinity effects were negligible (Landon, 1991). This result is particularly important for the *Enguti* area where irrigated production is practiced.

Based on the critical limits of DTPA-extractable micro-nutrient levels described in Motsara and Roy (2008), all soil samples from *Enerata* and *Akayita*, except samples taken from natural forest and homestead, had Zn content in the low range (0.5-1 mg kg^{-1}). Samples from eucalyptus plantations had the lowest Zn content at both sites. Samples from *Enguti* had medium Zn availability (1-3 mg kg^{-1}), the highest (2.9 mg kg^{-1}) being for homestead samples (Table 2.5). *Enerata* had a Cu content in the high range (0.8-3 mg kg^{-1}), the least being for eucalyptus plots and the highest being (2.3 mg kg^{-1}) for homestead soils. The most acidic environment, *Gashena Akayita,* had Cu availability mainly in the medium range (0.3-0.8 mg kg^{-1}). Only two samples taken from homestead soils (1.6 mg kg^{-1}) and eucalyptus plots (1.0 mg kg^{-1}) had Cu availability in the high range (0.8-3.0 mg kg^{-1}). Relative to *Enerata* and *Gashena Akayita*, samples from *Enguti* had greater higher Cu availability, the majority of the samples being in the medium range.

Iron contents for all the sites and the land-uses were within the very high range (>10 mg kg^{-1}). When the three sites are compared, iron availability was highest at *Gashena Akayita,* followed by *Enguti* and *Enerata*. Iron content of as high as 35.9 mg kg^{-1} was obtained at *Gashena Akayita*. Similarly, the Mn contents of *Enguti* and *Enerata* sites were in the very high (>6.0 mg kg^{-1}) range. Only two samples from homestead and natural forest soils at *Gashena Akayita* had a Mn content of >6.0 mg kg^{-1}. The rest of the samples from this site had Mn levels in the medium range (1.2-3.5 mg kg^{-1}). Unlimed outfields of *Enerata* and *Enguti* had $pH_{(H2O)}$ values of less than 5.5 and Mn content of >12.0 $mg.kg^{-1}$. Such a combination of low pH and extremely high Mn content could result in Mn toxicity to plants (Menzies, 2003). The widespread cultivation of oat and triticale on outfields of these areas could be associated with the tolerance of these species to Mn toxicity.

The farmers mentioned that their homestead soils were less acidic than the outfields, which were strongly acidic at all the study sites. Nonetheless, the area of homesteads often ranged between less than a quarter to one-third of the total land holdings of each household in all the study areas. The strongly acidic outfields constituted the bulk of the land available to each household across the study areas. Farmers were realistic in viewing soil acidity (from its symptoms) to be among the top five constraints affecting crop production across the study sites (Table 2.6).

Table 2.5. Chemical properties of the soils across study sites and predominant land-uses

Site	Land-use	PH (H2O) 1:2.5	PH (KCl) 1:2.5	ECe	Exchangable bases (cmol(+).kg^{-1}						% base Sat.	CEC cmol (+) .kg^{-1}	TN (%)	Av. P mg/Kg	Ex. Al cmol (+).kg^{-1}	Ex.acid cmol (+).kg^{-1}	Micro nutrients (mg.kg^{-1})			
					Ca	Mg	Na	K	Ca:Mg	K:Mg							Fe	Zn	Cu	Mn
Enerata	limed	5.64	4.38	0.29	20.26	3.14	0.1	0.5	6.45	0.16	69.36	34.6	0.04	3.24	0	0.48	13.2	0.7	1	14.4
	Unlimed	5.28	3.89	0.17	14.71	2.59	0.33	0.47	5.68	0.18	55.86	32.4	0.04	4.98	0	1.2	15.4	0.6	1.3	12
	Homestead	5.49	4.17	0.68	16.54	2.87	0.03	2.11	5.76	0.74	74.31	29	0.1	24.62	0	0.64	27.8	1.4	2.3	21.6
	Grazing land	5.36	3.95	0.23	17.16	3.45	0.03	0.65	4.97	0.19	68.68	31	0.1	4.21	0	1.12	21.1	0.7	1.5	16.9
	Eucalyptus	4.74	3.42	0.45	11.52	2.69	0.19	0.52	4.28	0.19	51.45	29	0.08	4.4	1.92	4.24	18.9	0.5	1	13.8
Akayita	limed	4.84	3.81	0.86	7.22	3.46	0.03	0.51	2.09	0.15	25.50	44	0.25	17.99	1.44	3.84	29.8	0.6	0.8	2.6
	Unlimed	4.57	3.73	1.24	1.56	6.93	0.08	0.47	0.23	0.07	21.02	43	0.27	18.82	1.6	4.08	28.3	0.7	0.7	2.6
	Homestead	5.16	4.12	1.68	12.16	0.76	0.07	1.52	16.0	2.00	38.18	38	0.25	34.6	1.76	4.4	39	1.5	1.6	7.9
	Grazing land	4.61	3.81	1.81	5.84	1.95	0	0.86	2.99	0.44	19.22	45	0.31	10.52	0.16	1.28	28.4	0.5	0.5	1.8
	Eucalyptus	4.82	3.81	0.86	6.09	6.64	0.09	0.35	0.92	0.05	30.63	43	0.06	13.67	1.36	3.36	27.6	0.4	0.4	1.5
	N. forest	5.44	4.62	1.43	33.74	4.75	0.08	0.95	7.10	0.20	73.19	54	0.76	3.18	0	0.48	35.9	1.6	1	7.1
Enguti	Unlimed	4.9	3.68	0.29	7.24	1.56	0.05	0.52	4.64	0.33	30.62	30.6	0.11	3.98	0.56	2.24	12.6	1.5	1.9	21.5
	Homestead	5.86	4.67	0.68	17.58	3.23	0.24	2.1	5.44	0.65	74.68	31	0.13	11.23	0	0.48	22.7	2.9	3.5	32.5
	Grazing land	5.23	4.07	0.83	17.19	4.58	0.24	1.72	3.75	0.38	66.66	35.6	0.18	3.56	0	0.64	32.8	1.7	2.5	17.1
	Eucalyptus	4.98	3.79	0.29	8.61	1.98	0.17	1.18	4.35	0.60	42.04	28.4	0.1	2.92	0.24	1.36	17.8	1.1	2.3	22.2
	N. forest	5.5	4.54	0.75	21.23	6.22	0.08	0.81	3.41	0.13	79.61	35.6	0.13	1.37	0	0.4	28.1	1.4	3	28.9

Table 2.6. Farmers ranking of of soil acidity in the top five constraints limiting crop production in the study sites

Priority	Study sites		
	Enerata (n=30)	*Gashena Akayita* (n=15)	*Enguti* (n=15)
1	Soil erosion, soil acidity and decline in soil fertility and prohibitive cost of fertilizer and lime and cash shortage	Lack of adaptable crop species and varieties	Soil acidity and prohibitive price of fertilizer and cash shortage
2	Erratic rainfall	Crop diseases	Shortage of labour
3	Crop diseases	Soil acidity and prohibitive cost of fertilizer and lime and cash shortage	Crop diseases and pests
4	Land shortage	Natural calamities: frost, hail and flooding	Land shortage
5	Lack of adaptable crop species and varieties	Land shortage	Lack of adaptable crop species and varieties for irrigated system

1-highest priority; 5- lower priority

2.3.4 Causes of soil acidity as perceived by farmers

2.3.4.1 Soil erosion and unwise farming

Farmers mentioned the loss of fertile top soil through runoff as one of the causes of soil acidity in the surveyed areas. Observations made during the surveys confirmed that soil erosion was more critical on cultivated, sloped outfields. However, this varied by location. Land degradation associated with soil erosion was more acute at *Enerata* than *Enguti* and *Gashena Akayita*. According to the farmers, rugged terrain, high rainfall, and cultivation of steep slopes with limited conservation practices were the primary underlying factors that aggravated soil erosion. Land degradation on grazing lands was driven by high animal populations and over-grazing, and the lack of any sustainable management system.

2.3.4.2 Contending use of animal manure and crop residue

According to the farmers, as the human population increased, the need for more agricultural land had resulted in extensive deforestation of the natural forest in the study areas. With a decline in forest cover, the resultant shortage of firewood forced the farmers to use cattle dung and stalks of crops like maize for fuel than to replenish the soil. During the dry season, dung dropped in homestead areas and grazing lands are systematically collected and dried for home use and sometimes for sale. Since

communal grazing lands were generally unproductive, farmers heavily relied on crop residue for animal feed.

Thatching for houses was another sink for stalks of small cereals such as wheat, barley and triticale that otherwise would be returned to the soil. Stalks of crops like wheat and triticale were preferred for roofing than animal feed. According to the CSA (2007), 54% of residential houses in the Amhara region were thatch roofed (Figure 2.3). Furthermore, almost all of residential houses in the rural areas and small towns were mud-walled, using a plaster consisting of mud and straw of small cereals such as tef and finger millet. The mud walls and the floors of these houses were also painted with cattle dung to make them smooth and good looking. Straws of tef, finger millet and other small cereals were also used for to create mattresses that are widely used in rural villages and small towns.

Grains, pastures and crop residues usually have an alkaline pH due to their high content of basic minerals (Upjohn *et al.*, 2005). Hence, the comprehensive outflow of basic minerals in the form of grain and crop residue, as seen in the study areas, will contribute to a growing decline in soil pH.

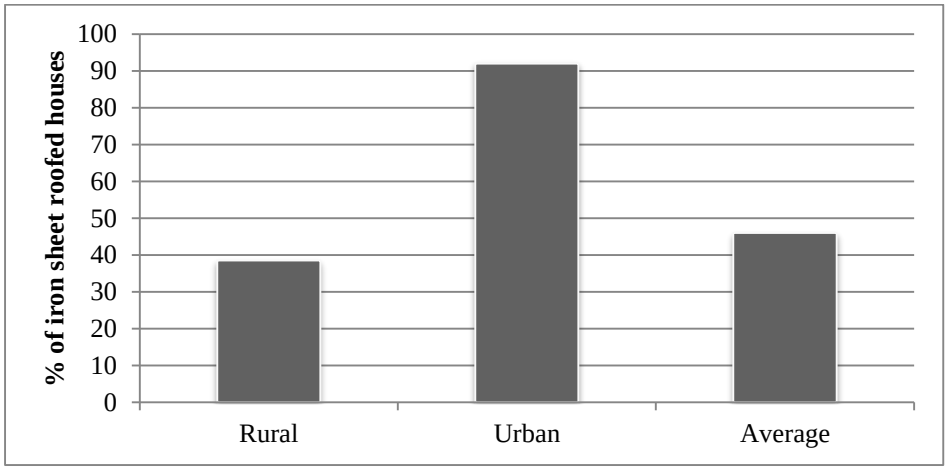

Figure 2.3. Proportion of corrugated iron sheet roofed houses for the Amhara region (Source: CSA, 2007)

2.3.4.3 Abandoning traditional fertility management practices

According to the farmers, high population pressures and the consequent shortage of arable land were the underlying factors leading to the abandoning of traditional fertility

management practices such as fallowing, '*Chichet*' or '*hura*' (corralling of cattle at night on outfields during the rainy season as a method of *in situ* manure application), crop rotation, and manure application. In the *Banja* area, where soil acidity is an acute problem, '*Chichet*' or '*hura*' is still maintained while it has been abandoned in *Enerata* and *Angti*.

According to the farmers, rotation of cereals with pulses and *noug* (*Guizotia abyssinica* (L.F.) Cass.) were traditional practices that had been primarily used to replenish soil fertility. However, the farmers stated that the suitability of outfields for the cultivation of pulses was declining due to the growing level of soil acidity. Consequently, the production of legumes was mainly restricted to the homestead areas, and less acidic outfields. Land shortages have also compelled farmers to give priority to the cultivation of cereals, which are staple foods and are needed for household food security. For household needs, pulses were intercropped with crops such as maize, potato and *Brassica* spp. on the homestead fields. Hence, cropping cereal after cereal, or rotation with the acid-tolerant white lupin have become common practices on the acidic outfields of the study areas

2.3.4.4 Limited use of external inputs

The farmers agreed that continuous and exploitative farming with little nutrient recycling characterized their crop production system. Di-ammonium phosphate (DAP) and urea accounted for 100% of the mineral fertilizers sold in the study areas for many years. DAP provides P and N while urea supplies only N. Consumption of grains and biomass concurrently remove basic cations such as K, Ca and Mg, in addition to N, P and other minerals (Upjohn *et al.*, 2005).

Application of fertilizers or lime was not optimal for all crops, across different land-uses and socio-economic groups. For instance, deliberate application of local or external inputs on communal grazing land is non-existent across all the study sites. Farmers in most of the study areas were reluctant to apply lime on acidic crop outfields due to its high initial cost, the costs of transporting lime, and the labour of applying it. Outfields were generally more acidic and were often fertilized with mineral fertilizer than homestead soils, which benefited more from manure and household refuse. Other reports have shown that the low level of fertilizer usage is one of the causes of depletion of soil nutrients in Ethiopian agriculture (IFPRI, 2010; ATA, 2013).

2.3.5 Indicators of soil acidity as perceived by the farmers

2.3.5.1 Land and soil characteristics

The farmers reported that sloped crop outfields, often with shallow soils, were the most affected by soil acidity, whereas bottomlands and flat plateau lands where the slope is close to zero were considered to be more fertile and less acidic. According to the farmers, the bottomlands benefit from the inflow of nutrient and organic matter through sedimentation from the uplands, whereas the flat plateau lands experience minimal outflow of nutrients through runoff.

Acidic outfields were described as '*kelal*' or '*forehe*' at *Enguti*. '*Kelal'* refers to their ease of ploughing and poor water holding capacity. '*Forehe*' refers to highly friable infertile soil lacking organic matter. Such soils were light red compared to reddish brown fertile outfields or deep brownish homestead soils. At *Enerata* such soils were called '*borebore*'. At *Gashena Akayita*, acid soils were described as '*gibiz*' which literarily means 'pretender'. Such soils appear to be fertile but in reality they were poor in crop response. Like the acid soils of *Enerata* and *Enguti*, *gibiz* soils were described as soils with good drainage and being easy to plough.

2.3.5.2 Plant growth and productivity attributes

Poor establishment, stunted growth, pale green young seedlings, poor stands and poor tillering by cereals and therefore, poor grain and straw yields were among the major indicators of acid soils mentioned by the farmers.

The diminishing suitability of the soils for the cultivation of once popular crops such as barley, faba bean and field pea, and the widespread cultivation of acid tolerant crops such as triticale, oat and white lupin was also another indicator of the increasing problem of soil acidity. At *Enerata* fields that were not suitable for production of even oat, triticale and lupin were described as "*Yemote*" or 'dead', to connote extremely acidic soils, which were often planted to eucalyptus. Farmers associated poor responses or an increasing demand of crops for mineral fertilizers with growing level of acidity.

2.3.5.3 Weed species

Prevalence of specific weed species on crop fields was also identified as another indicator of soil acidity. Farmers complained about prevalence of the weed couch grass (*Cynodon dactylon* (L.) Pers.*)* on the acidic outfields of *Enerata* and *Enguti*. At *Gashena Akayita,* corn spurry *(Spergula arvensis L.),* annual knawel (*Scleranthus annuus* L.), and tiny mousetail (*Myosurus minimus* L.) were the main weed indicators of soils with high levels of acidity. Poor pasture growth, often with slippery algal growth when wet, and the invasion by weed species of no feed value, were indicators of soil acidity on grazing lands.

2.3.5.4 Decline in crop genetic diversity

According to the farmers, a decline in area and diversity of once popular crop species, and the introduction of new crop and forestry species to the farming systems was mainly related to soil acidity. *Wofiye, Senefkolo, Limenish, and Werenj* were traditional barley cultivars that used to be widely grown but which were now under threat. At *Gashena Akayita, Sindemena, Temj, Saldini, Masno, and Dubar* were traditional barley cultivars that used to be grown widely. Among these, *Saldinin* and *Temeje* were disappearing most rapidly, due to the increasing levels of soil acidity. At *Enerata*, substantial areas of land that used to be covered by barley had been replaced by oat. The local name of oat is *Engido,* which was derived from the Amharic term *Engida,* which means “stranger” or “newcomer”. Currently, two cultivars of oat, *Chimburdi* and *Rejjimu engido*, are grown in the *Enerata* areas. Triticale is also another recently introduced, acid tolerant crop that is rapidly expanding and is replacing the traditional crops.

Farmers at *Enerata* area identified *Dabo, Tikurmure, Bursa* or *Sergegna*, *Zambi, Natchmure* as landraces of tef grown in the area. Currently, *Dabo* is the most popular and widely grown landrace. It is liked for its adaptation to the soil and its earliness. Nonetheless, this landrace is brown seeded and fetches a lower price on market than white seeded varieties. It is also short statured, which means that it provides little straw and is difficult to harvest. According to the farmers, there has been a decline in production of white seeded and *bursa or* mixed-colour landraces in their area due to their poor tolerance to acid soils. A similar pattern was also witnessed by farmers from

Gashena Akayita. The effect of soil acidity on crop genetic resources diversity is pronounced in the highland areas, *Enerata* and *Gashena Akayita,* where the range of crop choices is already limited due to the low temperatures prevalent in these high altitude districts.

The rate of conversion of crop lands to eucalyptus and green wattle (*Acacia decurrens* (Wendl.) Willd.) was alarming and a threat to the remaining genetic resources in the wild and crop environment. Farmers reported a substantial loss of genetic resources as a result of agricultural encroachment onto the areas remaining of natural vegetation, and the selection pressure exerted by soil acidity. The contribution of natural forests to the overall vegetation of the study areas has been significantly reduced during the last half a century, and acid tolerant, exotic forestry species such as eucalyptus now covers much of the study area. Concurrently, the communal grazing lands have also been overgrazed, and have become extremely acid, with the result that they have been invaded with weed species of no feed value that can thrive well on acidic soils.

2.3.6 Coping strategies

The coping strategies of the farmers to deal with soil acidity were classified into two categories. The first were the strategies being promoted by the extension service which included recommendation to the farmers that they apply lime, mineral fertilizer and compost, and that they should diligently implement soil and water conservation measures. Shifting to production of tolerant crop species, landraces and forestry species; night corralling of cattle and manure application; spatial segregation of crop species and crop rotation were the farmers' own coping strategies. However, the viability and hence the levels of implementation of these coping strategies was determined by various socio-economic and technical factors (Table 2.7).

2.3.6.1 Application of lime

Despite the high prevalence of acid soils in the study areas, acid soil reclamation by the application of lime had only started relatively recently, in 2007. Even so, lime utilization has been relatively insignificant. For instance, the quantity of lime utilized in the most acidic district of the study areas, *Banja*, over a period of 7 years was only

433.5 t. This was equivalent to a mean use of only 62 t per annum for an area of over 12,000 ha that had been cultivated annually, a mere 5.2 kg ha^{-1} (Figure 2.4).

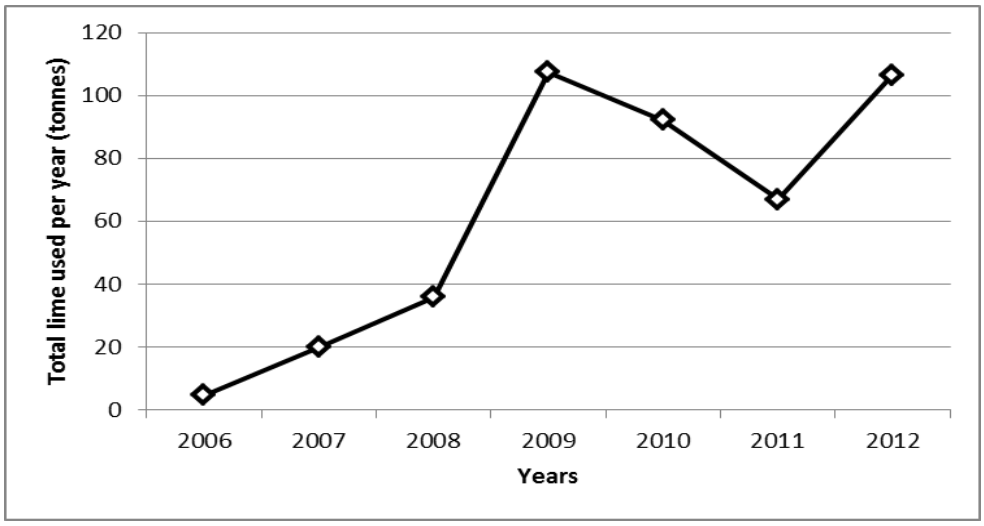

Figure 2.4 Lime utilization pattern for the most acidic district, *Banja*, in the years 2006-2012 (Source: Awi Zone Office of Agricultural Development)

Various factors constrained lime use in the study areas. Lime recommendation were as much as 16 $t.ha^{-1}$ in the study areas. Extremely low pH (KCl) of below 4.0 for all unlimed samples, coupled with high and medium CEC, reflected the strong buffering capacity of the soils. Liming of such soils needs large quantities of lime to neutralize the acidic cations (H^+ and Al^{3+}) in soil solution, as well as on exchange sites (Landon, 1991; Rao *et al.*, 1993).

According to the farmers and key informants, the farmers had no cash for purchase of crop inputs because of the low productivity and low market value of the crops grown in the study areas. Thus there was no possibility of the farmers buying the large quantities of lime that were recommended to combat the soil acidity. The problem was further aggravated by the lack of all-weather roads, the absence of farm roads, the ruggedness of the terrain, and the fragmentation of each farmer's lands. Risks of crop losses associated with hail, frost, flood, pests and diseases were other factors that made farmers unwilling to gamble on the use of lime, even when a combination of credit and a subsidy was available to the farmers in order to buy lime. Farmers also

complained about a perceived health risk associated with manual application of lime, applied as a fine dust.

2.3.6.2 Mineral fertilizers

Due to their high level of acidity, outfields were always given priority for mineral fertilizer application. Mineral fertilizers were applied at recommended rates or higher levels on crops such as maize at *Mecha* and wheat at *Banja* and *Gozamin*. The farmers reported that all the outfields needed mineral fertilizer applications to give reasonable yields of all crops. Soaring fertilizer prices, the poor financial status of the farmers, the unavailability of crop insurance, the high risks posed by hail, frost, pests and disease damage, and prohibitive interest rates were among the factors that stopped farmers from using the local credit services available to them. Consequently, most of the resource poor farmers applied sub-optimal rates of mineral fertilizer, or resorted to other options.

Diammonium phosphate (DAP) and urea are two mineral fertilizers that have been applied exclusively on all soil types and in all agroecologies in Ethiopia. The farmers expressed their discontent with the use of these mineral fertilizers, describing them as ‘addictive’, in that the soil needs increasing quantities of these fertilizers, season after season. The farmers firmly believed that, “these mineral fertilizers have spoiled our soil”. The assimilation of these fertilizers in roots produces protons that are released to the external medium and thereby increase rhizosphere acidity. Furthermore, leaching of nitrate, converted from these N sources, along with basic cations increases root zone acidity (Marschner, 1995; Barak *et al.*, 1997; Bolan and Hedley, 2003). Declining responses of crops to recommended fertilizer levels as reported by the farmers would also be caused by the acidifying effect of these mineral fertilizers. Thus, these fertilizers should not be applied in areas with acid soils without concurrent applications of acid equivalent quantities of lime that can neutralize the acidity released from the fertilizer material alone (Bolan and Hedley, 2003). Utilization of non-acid forming fertilizers in areas affected by acid soils is a better option for resource poor farmers who cannot invest more on external inputs, and lime in particular (Bolan and Hedley, 2003).

2.3.6.3 Compost and manure

Compost preparation and utilization at the household level has been strongly promoted by the extension service in order to improve soil fertility. Use of organic matter in the form of manure and compost can reduce soil acidity (Haynes and Mokolobate, 2001; Wong and Swift, 2003). However a number of factors work against this approach. These include: A shortage of labour to collect and apply the materials; difficulties in transporting large quantities of compost to outfields in the absence of roads or tractors; competing use of animal manure for fuel; and the use of green matter for animal feed (Schlede, 1989; IFPRI, 2010). Consequently, compost preparation is limited to the rainy season when there is an adequate supply of manure, green matter and moisture. Its application was also mainly restricted to the homestead areas.

2.3.6.4 Soil and water conservation (SWC)

The farmers indicated that soil acidity was worse on sloped outfields than bottom lands, flat plateaus and homesteads. Soil erosion was an active and widespread sign of physical land degradation that has captured the attention of high level policy makers and experts. Consequently, extensive work was being done on soil and water conservation (SWC) measures in the study areas through mass mobilization of the population under the supervision of national experts. The objective of soil conservation practices was to lessen the extent of soil and water loss through runoff, and to improve crop productivity through the optimal use of mineral fertilizers and compost. Research has shown that water runoff removes basic cations, including liming materials, and that it accelerates the rate of acidity development (Ritchey *et al.*, 2012).

2.3.6.5 Shifting to production of adapted crop species and acid-tolerant landraces

At *Gashena Akayita,* which was the most acidic environment, a lack of adaptable and high yielding cultivars was reported to be the most important constraint of crop production (Table 2.6). Key informants also confirmed that the performance of the "improved" varieties of wheat, tef and other crops at *Gashena Akayita* and *Enerata* was poor. Consequently, farmers had shifted to growing brown seeded tef landraces, potato, triticale, oat, lupin, and timber crops. These crops are well adapted to acid soils. According to the farmers in *Enerata* and *Gashena Akayita*, white lupin is valued

because it tolerates soil acidity and it improves soil fertility, helping to avoid or reduce the need to apply mineral fertilizer on subsequent crops such as wheat. However, because of land shortage and its negligible value as a food and feed crop, only farmers with large areas of farming lands could afford to include lupin in their rotation or intercropping system. The lupin cultivars were exclusively high alkaloid types and were not used for forage. Hence, the main benefit of lupin is from biological nitrogen fixation by rhizobia, as well as the minerals and organic carbon released by the decomposition of its biomass after harvest.

2.3.6.6 Spatial segregation, rotation and others

The farmers broadly categorised their crop production fields into two classes, based suitability for crop growth. The first was called '*lem*' '*kilze*' '*yebadima afer'* or '*yeguaro afer*' and represented the fertile soils that were mainly located around the homesteads and the bottom lands. The second was '*borebor' (Enerata)* or '*Kelal*' or '*forehe*' at (*Enguti*) or '*Gibiz*' at *Gashena Akayita*, which represented acidic outfields. In the study areas, the various crops were spatially segregated, based on the sensitivity of each crop to soil acidity. At *Gashena Akayita*, which was the most acidic environment, cultivation of acid sensitive crops was restricted to the homestead areas, which had the least acidic soils. Heavy feeder crops such as wheat were grown on relatively fertile and less acidic outfields with the application of mineral fertilizers at *Gashena Akayita*, often following a lupin fallow in the *Enerata* area. Oat and triticale were produced on acidic outfields without mineral fertilization. Tef and finger millet were cropped with sub-optimal applications of mineral fertilizers on outfields.

According to the farmers, increases in level of soil acidity had minimized the role of rotation crops in their farming systems. The cultivation and productivity of legumes such as faba bean and field pea on outfields had substantially decreased with increasing level of soil acidity. The decline in production of these legumes can be associated to poor adaptability of these legumes and their strains of *Rhizobium* to acid soils (von Uexkull, 1986; Miyasaka *et al.*, 2007). Nodulation by *Rhizobium* is also affected by Al and Mn toxicities, and deficiencies of P (Caradus, 1993). Since legumes are also sensitive to Zn deficiency, the low level of Zn (<1.0 mg kg^{-1}) in the most acidic environments would compound the problem (Sillanpaa, 1972).

White lupin, noug, and linseed (*Linum usitatissimum* L.) were the main rotation crops grown on the outfields. The common rotation crop cycle in the *Enerata* area was noug / linseed / oat / lupin, followed by cereals (tef / wheat / triticale). In *Gashena Akayita* and the neighbouring highlands, most rotations included potato and cereals, but left out lupin. In the *Enguti* area, noug and occasionally linseed were typically rotated with cereals. However, due to a decline in the productivity of beneficial rotation crops, and because of land shortages, the cropping of cereal after cereals was becoming a common practice.

2.3.6.7 Shifting to forestry and livestock

Eucalyptus was the dominant vegetation cover of the study areas. The increasing levels and spread of soil acidity, and the prohibitive cost of mineral fertilizers and lime were the primary factors driving the planting of eucalyptus in the study areas. Eucalyptus is highly tolerant to Al-toxicity (Neves *et al.*, 1982; Barros and Novais, 1996). The planting of eucalyptus plantations has a number of advantages. The timber can be sold in various forms and can generate a good income. Its primary use is for fuel and building materials. The crop is tolerant of acid soils, drought, hail, diseases and pests and does not need labour for routine management, or fertilizer applications. Furthermore, timber crops are a recognized capital asset and can also be used as collateral to borrow money from informal sources. A further advantage is that, after the planting of eucalyptus trees, no further management is needed, allowing male members of the households to migrate to urban areas to seek informal labour in order to generate a secondary income.

In addition to eucalyptus, farmers in *Banja* and neighbouring areas with acid soils were switching production in their outfields to green wattle (*A. decurrens)* plantations. This timber crop has multiple uses, especially as a feedstock for charcoal production by the farmers. Green wattle is a nitrogen-fixing tree species (Roughley, 1986) and its ectomycorrhizal association has also been reported (Reddell and Warren, 1986). There is little information on its tolerance to acid soils, however, its luxuriant growth in the study areas suggests that it is highly tolerant of soil acidity.

2.3.6.8 Corralling of cattle at night on crop or grazing lands

There was a traditional practice of corralling of cattle on crop land, with the objective of replenishing soil fertility through the direct application of dung and urine. This was known as “hura” in the *Banja* area, and “*chihit*” in the *Gozamin* and *Enguti* areas. It was a longstanding traditional soil fertility management that persisted mainly in *Banja* and other districts of the Awi Zone in north western Ethiopia. A similar practice has been reported in several African countries, where it is also used to maintain soil fertility (Murwira *et al.*, 1993; Harris, 2002). Compared to manure collected from pens or compost, corralling of livestock at night on crop lands does not demand labour for collection, storage, preparation, transporting and application. This is particularly important because a single farmer’s outfields are often fragmented and scattered. *‘Hura’* is practiced during the wet season when cattle provided greater volumes of dung and urine, and when there is little loss of nutrients as a result of solar radiation, heat or drying winds. One challenge is that *‘Hura’* needs the collective action of farmers in a village due to the relatively few cattle held by each household.

Table 2.7. Summary of farmers’ assessment of constraints associated with soil fertility management methods at the study areas.

Management method	Contending use	Poor yield response	Has no long term effect	Cash shortage	Difficulty to transport	Difficulty to apply	Labour demanding	Land shortage	Needs collective action	Technical problems	Limited crop choice
Mineral fertilizer			*	*							
Compost	*				*		*			*	
Lime		*			*	*	*			*	
Animal manure	*			*	*		*				
Rotation		*	*					*			*
Short fallow								*			
Night corralling	*							*	*		
Erosion control		*						*	*		

2.3.6.9 Other coping strategies

According to farmers and key informants, conversion of most acidic crop outfields to private pastures was an increasing trend in the *Banja* area as a strategy to cope with soil acidity. Resource poor farmers who could not afford to purchase mineral fertilizers and lime rented out or share-cropped their outfields to better-off farmers.

2.4 Discussion

The farming systems of all the study areas can be classified as a highland temperate mixed farming system, as described by Dixon *et al.* (2001). By virtue of their suitability for human and animal health, the distribution of the human population is generally skewed towards the highland areas of the world (UNECA, 1996; Pankhurt, 2009). Human population density for all the Districts in the study was higher than the national mean, being 85 people km^{-2} (http://country-facts.findthebest.com/l/84/Ethiopia), and the mean land holding was below 2.0 ha (Dixon *et al.*, 2001). Land degradation associated with soil erosion and nutrient depletion is a serious problem affecting this farming system (Schlede, 1989; Dixon *et al.*, 2001; Dubale, 2001; Bishaw, 2001; IFPRI, 2010).

Soil test results confirmed the prevalence of soil acidity across all the study areas and land-uses. Despite their clay and heavy clay textures, the overall CEC values of the soils of the *Enerata* and *Enguti* sites were lower than those of *Gashena Akayita*. This can be partly attributed to a relatively high organic matter content in soils from *Gashena Akayita*. Compared to organic matter, the contribution of clay minerals to CEC is extremely low (Landon, 1991). The higher exchangeable acidity at *Gashena Akayita* indicated a greater contribution of acidic cations Al^{3+} and H^{+} to the CEC of this site. Such soils need the application of large quantities of lime to neutralize the high levels of Al^{3+} and H^{+} ions in the soil solution, as well as on the exchange sites. But this high rate was prohibitively expensive for small-scale farmers due to their financial and technological limitations (Rao *et al.*, 1993). Most of the farmers in the study areas were not willing to apply lime to their soils because of the cost factor. Exchangeable Al of 2-3.0 cmol kg^{-1} of soil is excessive for some crop species (Chapman, 1966). As the soil samples were composites, high levels of exchangeable Al are expected, particularly from the acidic Acrisols of *Gashena Akayita*. The crop outfields from *Enerata* and *Enguti* had $pH_{(H2O)}$ levels of less than 5.5 and Mn levels of >12.0 mg kg^{-1}. Such combinations of low pH and extremely high Mn content would result in Mn toxicity for most plants (Menzies, 2003). When the pH falls below 5.0, Mn toxicity occurs concurrently with Al toxicity. However, tolerant plants can change the toxic divalent manganese to a non-toxic form through increase of the rhizosphere pH, hence bulk soil data may not reflect the toxicity of Mn on roots (Menzies, 2003).

The variability observed in the balance of the bases and micronutrient levels across the various land-uses and study areas could be associated with intrinsic and extrinsic factors. For instance, the Nitosols of *Enguti* and *Enerata* showed similar patterns for available P and potentially toxic levels of Mn. While *Gashena Akayita* represented the sub-humid climate, the Acrisols of *Banja* and its neighbouring districts were distinct. Unique and ancient management practices such as *'hura'* or corralling of livestock on crop lands at night, was widely practiced in the *Gashena Akayita*, and would have contributed to the higher level of available P measured there. At *Gashena Akayita*, exchangeable Al^{3+} was detected from more of the land-uses than the other two sites. The contributions of both intrinsic and anthropogenic activities to variations in important chemical properties has also been reported for acid soils in India (Behera and Shukla, 2015). The worst soils, with poor availability of nutrients, were in the outfields and eucalyptus plots. Crop production on outfields was difficult without N application. However, the N sources being used were exacerbating the problem. In addition to the problems of N deficiency and P fixation, the imbalanced levels of Ca and the unavailability of Ca were also a problem for crop production on the outfields of the study areas. Hence, the application of calcium ammonium nitrate 20% N and 6% Ca or calcium nitrate urea (calurea, 34% N, 10% Ca), along with other P sources such as superphosphate, can be recommended to provide N, P and Ca without enhancing the soil fertility problems of the study areas (Barak *et al.*, 1997; Bolan and Hedley, 2003).

Farmers and key informants identified soil erosion, and unwise farming practices; poor nutrient recycling, and competing uses of animal manure and crop residues; the abandoning of traditional fertility management practices; and the limited use of external inputs as the major causes of soil acidity. High rainfall, undulating land profiles, the poor water holding capacity of the soil, and inadequate soil and water conservation practices have contributed to the severe loss of soil in the highlands of Ethiopia (Lakew *et al.*, 2000; Bishaw, 2001). Controlled experiments have shown that runoff removes basic cations including liming materials and accelerates the rate of soil acidity development (Anna *et al.*, 1997; Ritchey *et al.*, 2012). In Ethiopia, cultivated outfields are more seriously affected by soil erosion than grazing land (Bishaw, 2001). Hence, the high level of soil acidity on crop outfields revealed in this study can be partly explained by high levels of soil erosion. Due to their distance from residential

areas and the difficulties of transporting compost and manure, outfields were the worst affected by the poor recycling of basic cations in the form of organic matter (Teshome *et al.*, 2014). Low organic matter content in soils of all the land-uses in the study areas would also contribute to poor soil physical properties and would enhances the loss of basic cations through soil erosion and leaching.

The widespread utilization of crop residues for animal feed and thatching, and the use of animal manure for fuel were the main competing uses of organic matter that would otherwise be used to replenish soil fertility. Organic matter content of all the dominant land-uses across all the study areas was low to extremely low. Similar results were obtained in a previous study conducted the study areas (Yihenew, 2002). In the mixed farming system of the central part of Ethiopia, 70% of the total tef straw produced is used for animal feed (Zinash and Seyoum, 1991). Grain, pasture and crop residues generally have an alkaline pH due to their high content of basic minerals (Upjohn *et al.*, 2005). Hence, continuous removal of basic minerals in the form of grains and biomass subjects crop and grazing lands to increasing soil acidity (Murwira *et al.*, 1993). Widespread use of cattle dung for fuel in the mixed farming system of Ethiopia has been reported in several studies (Schlede, 1989; Dixon *et al.*, 2001; Dubale, 2001; Bishaw, 2001; IFPRI, 2010). Zenebe (2007) estimated that the use of dung as fuel instead of fertilizer reduces the country's agricultural GDP by 7.0%. The beneficial effects of manure for soil fertility is mainly related to its supply of P, basic cations such as Ca and Mg, organic matter and its contribution to the improvement of soil physical properties (Murwira *et al.*, 1993; Giller *et al.*, 1996). Increasing pressure on agricultural land has also compelled farmers to abandon traditional soil fertility replenishment practices such as fallowing, night-corralling, crop rotation etc. resulting in depletion of soil nutrients (Sanchez *et al.*, 1997; Lakew *et al.*, 2000).

Farmers did not recognize that leaching associated with high rainfall, and the soil parent material, were the major causes of the decline of soil fertility or the development of soil acidity. Soils in high rainfall and high temperature areas acidify faster because of high rates of weathering and the leaching of basic cations (Hede *et al.*, 2001). The amount of rainfall in the study areas is generally high and consistent. Specifically, the sub-humid agro-ecology of *Banja* (*Gashena Akayita*) receives over 2000 mm per annum, whereas *Mecha* (*Enguti*) and *Gozamin* (*Enerata*) receive above 1200 m

(IFPRI and CSA, 2006). The higher levels of soil acidity at *Gashena Akayita* than the other two study sites relates mainly to the district's higher rainfall (Figure 2.2).

Leaching of basic cations can be high in clay minerals that are dominated by a 1:1 silicate layer such as kaolinite, which does not fix significant quantities of basic cations when compared to montmorillonitic or other 2:1 group clay particles (von Uexkull, 1986). The soils of *Enguti* and *Enerata* are predominantly Nitosols while that of *Gashena Akayita* is an Acrisol (Yihenew, 2002; IFPRI and CSA, 2006). Inherently, the clay assemblages of these two soil classes are dominated by the kaolinite (Driessen *et al.*, 2001). Hence, these soils lose basic cations rapidly through leaching and hence acidify faster than soils with less drainage.

Neither farmers nor key informants recognized that the mineral fertilizers being used enhanced the development of soil acidity. However, the farmers said that 'mineral fertilizers are addictive and have already spoiled our soils'. This can be related to increasing levels of acidity as a result of the acidifying effects of DAP and urea which are considered to be acid forming fertilizers. Assimilation of these fertilizers results in the release of H^+ in to the rhizosphere, increasing soil acidity (Marschner, 1995; Barak *et al.*, 1997; Bolan and Hedley, 2003). The fact that the outfields had lower pH values than other land-use areas can be ascribed to the acidifying effect of these fertilizers being applied to the crop lands.

Farmers used various terms that indicated physico-chemical and plant attributes that related to acid soils. As the loss of basic cations through erosion is enhanced with an increase in slope of lands, the identification of slope as a cause of soil acidity by the farmers was valid. Abebe (2007) also found that acid soils were found on gentle to steep slopes of western, north western, south western and southern parts of Ethiopia.

As manure and compost utilization is labour demanding, they are often applied on gardens in mixed farming systems of small-scale farmers in Africa, but not to large cereal fields (Giller *et al.*, 1996; Sanginga and Woomer, 2009). Teshome *et al.* (2014) also reported similar practices in north western Ethiopia. Consequently, garden or homestead soils were presumed to be in a suitable pH range for crop growth in the study areas. Nonetheless, in the most acidic environment, *Gashena Akayita*, the pH of the homestead soils was below 5.5, with high levels of exchangeable Al, suggesting the need to reconsider the current assumption and to formulate suitable management

options. Various indicators of acid soils were identified by farmers: Poor crop stands, stunted growth, reduced microbial activity, high friability of the soils when ploughing, poor water holding capacity (specifically of the Acrisols of *Gashena Akayita*), all of which are scientifically valid (Little, 1989; Driessen *et al.*, 2001; Upjohn *et al.*, 2005). Corn spurry (*Spergula arvensis* L.) and annual knawel (*Scleranthus annuus* L.) are acidophilic weed species that constrain crop production in areas with acid soils (Čiuberkis, 2001; Čiuberkis and Koncius, 2006). A strong correlation between farmers' indicator plants of soil fertility with soil analysis results has been reported previously (Karltun *et al.*, 2013). Hence, the use of these weeds as markers of acidic soils by farmers was also valid.

Changes in the spectrum of crops grown on agricultural lands followed increases in soil acidity. Such trends can be associated with the variable sensitivity of plant species to low pH soils and their associated mineral toxicities (von Uexkull, 1986; Rao *et al.*, 1993; Upjohn *et al.*, 2005). The declining role of legumes such as field pea and faba bean in the rotation systems of the study areas could be associated their intolerance of soil acidity, and Al and Mn toxicities, that affect both the host and their symbiont *Rhizobium* strains (Hamdi, 1982; von Uexkull, 1986; Upjohn *et al.*, 2005). In addition to soil acidity, overgrazing can also change species composition on grazing lands (Angassa, 2014).

Soil acidity selects acidophilic plants that have the capacity to grow on acid soils due to their peculiar capacity to overcome Al-toxicity and low P availability (Houdijk *et al.*, 1993; Roem and Berendse, 2000). This selection pressure results in the loss of calcicole species and variants in wild and cultivated areas. Oat has been recognized to have replaced a wide range of local crop species and landraces in the farming systems of the central highlands of Ethiopia (IBC, 2007). A rapid expansion of triticale production in the study areas is also causing the loss of indigenous crop genetic resources.

On strongly acidic crop outfields, a switch from crop production to eucalyptus plantations is one of the most prevalent coping strategies adopted by farmers to deal with severe soil acidity. Eucalyptus is highly tolerant of acid soils and aluminium toxicity (Neves *et al.*, 1982; Barros and Novais, 1996). Leite *et al.* (2010) reported reductions in the exchangeable Ca^{2+}, Mg^{2+} and K^{+} and increases in Al^{3+} and H^{+} contents as a consequence of eucalyptus cultivation. The soil samples collected from

eucalyptus plantations in this study were highly acidic and had high levels of exchangeable Al, confirming that eucalyptus cultivation can enhance soil acidity.

Finally, the loss of productivity of crop and grazing lands due to increasing soil acidity has forced farmers to open up new lands by encroaching on the remnants of the wild ecosystem, decreasing the biodiversity associated with the wild ecosystem (Sanchez, 1995).

2.5 Conclusion

The soil samples showed variability in the physicochemical properties across study areas and land-uses. Such variation was related to intrinsic factors such parent material and climate, as well as to anthropogenic activities such as land-use and management practices. Most of the soil samples gave strongly acidic reaction. Soil samples from eucalyptus plantations, crop outfields, and grazing lands were the most acidic, with high levels of exchangeable Al. Among the study areas, *Gashena Akayita* was the most acidic environment, and even the homestead soils gave a strongly acidic reaction.

Farmers ranked soil acidity as one of the top five production constraints. Farmers perceived soil acidity to result from a range of causes that emanated from increasing densities of humans and animals. Although climate (high rainfall), edaphic factors, and the use of acid forming fertilizers were major contributing factor to soil acidity, they were not recognized by farmers and key informants as being directly associated with soil acidity.Farmers utilized various indicators of soil acidity to classify their crop fields, most of which were correlated with the actual presence of soil acidity and related soil conditions.

The indigenous coping strategies used by the farmers and those promoted by the extension were not compatible due to various socioeconomic and technical constraints. In particular, the value of most of the crops grown in the most acidic environments were low. Consequently, the farmers' capacity to invest on external inputs was limited. Furthermore, the costs of the proposed investments in lime and fertilizer would not be matched by the financial returns the crops could provide. The low pH and the high buffering capacity of the acid soils meant that large quantities of lime rates were needed. And the only fertilizers used in the region are acid-forming fertilizers that exacerbate the problem. In addition, the "improved" crop varieties

released to the farmers were not bred for tolerance to soil acidity. Hence, farmers have resorted to the production of unpopular, and low yielding crops and landraces that fetch little income but reliably produce crops on acid soils. The predominance of inherently acid tolerant crop species and landraces in the farming system demonstrated the significance of soil acidity in determining crop choices. Uncontrolled conversion of crop lands to forestry species was changing the farming system from a crop-livestock farming to a crop-livestock-forestry system, with the largest area devoted to forestry. Such a shift can compromise food production and challenge food sovereignty at the household level and beyond. So far, little research has focused on farmer-friendly of management strategies to deal with acid soils. Nor has there been studies or recognition of a range of farmers' coping strategies. With the current resources available to the farmers, sustainable management of acid soils and improvement in productivity of the system seems very unlikely. Hence, the development of technologies compatible with smallholders' system are needed. Such options need to combine the philosophies of both "changing the plants to fit the soil" and "changing the soil to fit the plant (Schaffert, 1993).

Towards this end, there is a need to emulate the farmers' indigenous coping strategy in developing improved varieties of acid tolerant crops, forages, and forestry species as components of a set of sustainable acid soil management technologies to be used across the different land-uses. It is also imperative that there is a nationally coordinated programme to rescue priceless crop genetic resources under threat of extinction in the regions of the country with acid to highly acid soils.

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CHAPTER 3

[3]Preliminary investigation on presence of genetic variability for soil acidity in tef [*Eragrostis tef* (Zucc.) Trotter]

Abstract

Tef [*Eragrostis tef* (Zucc.) Trotter] is the most widely produced and consumed cereal crop in Ethiopia. It is a gluten free crop with growing popularity worldwide. Unlike most globally important cereals, tef has not yet been bred for tolerance to soil acidity and to Al-toxicity. This study was conducted to assess the quantitative responses of some grain and pasture varieties of tef to soil acidity. A strongly acidic soil (pH 3.94 and an acid saturation of 78%) was used to evaluate the tef varieties. A highly Al-tolerant weeping lovegrass [*Eragrostis curvula* (Schrad.) Nees], variety, Ermelo, was used as a check. A randomized complete blocks design (RCBD) with 4 replications was used to evaluate the materials under limed and unlimed conditions. Measurements were taken of various root and shoot parameters. The results indicated the presence of genetic variability among the tef varieties for root length, shoot length, root dry weight and shoot dry weight. All the tef varieties were inferior to the *E. curvula* var. Ermelo in their Al-tolerance. The brown seed tef varieties consistently showed better Al-tolerance than the white seeded varieties. A similar pattern was also observed for tolerance indices, which were computed as the ratio of the value under unlimed to limed condition. Highly significant correlations ($r>0.9$) were observed for all the parameters used to assess Al-tolerance in this experiment. This is the first systematic study to demonstrate the presence of genetic variability for soil acidity and Al-tolerance within *E. tef*.

Key words: Aluminium toxicity, *Eragrostis tef*, genetic variability, screening, soil acidity

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3.1 Introduction

Acid soils (soils with a pH < 5.5 in the surface layer) constitute 3,950 million ha or 30% of the world's total ice-free land. In Africa, 22% or 659 million ha of the total 3.01 billion ha land has a soil acidity problem ((von Uexk¨ull and Mutert, 1995; Malcolm and Andrew, 2003) . The main problems of crop production on acid soils is mineral toxicity related to aluminium, manganese, and iron, and deficiencies of phosphorus, calcium, magnesium, and molybdenum ((von Uexk¨ull and Mutert, 1995; Hede *et al.*, 2001; Kochian *et al.*, 2004). Sixty-seven percent of the acid soils of the world have Al-toxicity problem ((Eswaran *et al.*, 1997).

In Ethiopia, acidity-related soil fertility problems are major production constraints, reducing productivity of the major crops grown in the country (Dubale, 2001; IFPRI, 2010). The soil acidity problem of Ethiopia is mainly related to the Oxisols and Ultisols soil classes, and some Alfisols, that occur in the western, north-western, south-western and southern parts of the country ((Abebe, 2007).

Tef [*Eragrostis tef* (Zucc.) Trotter] is the most widely produced and consumed cereal crop in Ethiopia (Spaenij-Dekking *et al.*, 2005). Tef is routinely cultivated on about 3 million hectares or 30 of the total area covered by cereals in the country (CSA, 2015). Consequently, it is among the worst affected crops by soil acidity. Tef responded poorly to fertilizer application on acid soils (Mamo and Killham, 1987; Mamo *et al.*, 1996; Spaenij-Dekking *et al.*, 2005).

Beyond Ethiopia, countries such as Eritrea, USA, the Netherlands and Israel produce a negligible quantity of tef as a grain crop (Spaenij-Dekking *et al.*, 2005). On the other hand, South Africa, India, Pakistan, Australia, Uganda, Kenya and Mozambique grow tef mainly as a forage or pasture crop (Assefa *et al.*, 2010).

The use of lime, compost, manure and other organic fertilizer sources has been recommended to cope with problem of soil acidity. However, these options are constrained by several factors. In the tropics, most acid soils have a strong buffering capacity against amendments of lime (Rao *et al.*, 1993). Hence, large amounts of lime are needed to normalize the pH. Most resource-poor farmers in the tropics are constrained by the local unavailability of lime, the high cost of transport and the unaffordable costs of the large quantities needed to treat the soils (Rao *et al.*, 1993; von Uexk¨ull and Mutert, 1995). In addition, lime has low mobility and its mechanical

incorporation into the subsoil is difficult for farmers without large tractors. Consequently, when surface soils are amended with lime in adequately, the failure to adjust the pH of the sub-soils results in restricted root growth and, therefore, poor plant growth (Rao *et al.*, 1993; von Uexk¨ull and Mutert, 1995; Abebe, 2007). Limited root growth also increases the vulnerability of plants to drought of even short durations (Foy, 1992). This is particularly important because many acid soils have inherently low water holding capacity (Little, 1989; Haynes and Mokolobate, 2001). The use of organic matter in the form of manure and compost can significantly reduce soil acidity (Wong and Swift, 2003). However, in countries like Ethiopia animal manure and crop residues are heavily used as fuel and animal feed, respectively, with the result that this option is not commonly applied (Schlede, 1989; IFPRI, 2010). The problem of soil acidity in cultivated land is further aggravated by the use of acid-forming chemical fertilizers. The predominant inorganic fertilizers available in Ethiopia are urea and diammonium phosphate (DAP) (Abebe, 2007). These fertilisers increase soil acidity when converted to nitrate nitrogen by releasing hydrogen ions (Barker and Bryson, 2007).

Worldwide, development of varieties tolerant to aluminium has been a sound alternative to liming and other non-genetic management options in the production of globally important crops such as wheat, rice, maize, barley, sorghum and rye (Foy and Murray, 1998; Pinto-Carnide and Guedes-Pinto, 1999; Hede *et al.*, 2001; Paterniani and Furlani, 2002; Kochian *et al.*, 2005; Portaluppi *et al.*, 2010). On tef, no systematic study has been made searching for tolerance to Al-toxicity. However, a closely related forage species, weeping lovegrass [*Eragrostis curvula* (Schrad.) Nees], is known to have a high level of tolerance to soil acidity (Miles and de Villiers, 1989). This species is considered as one of progenitors of tef (Ketema, 1993). This research work was conducted in order to investigate the presence of genetic variability among some grain and pasture varieties of tef.

3.2 Material and methods

3.2.1 Genetic stock

Four grain and 5 pasture tef (*E .tef*) varieties, along with *E. curvula* var. Ermelo, were evaluated under greenhouse conditions at the University of KwaZulu-Natal, Pietermaritzburg, South Africa.

3.2.2 Experimental set up

A sample of highly acidic subsoil with a pH of (KCl) 3.94 and an acid saturation of 78% was used in the experiment. The acid soil was analysed for pH and other chemical properties at the Soil Fertility Analytical Services laboratory of the KwaZulu-Natal Department of Agricultural and Environmental Affairs (Table 3.1). The soil was limed to a pH of 6.21 (KCl) with the application of 3.6 g of $CaCO_3$ (97%) powder per kilogram of dry soil and was incubated for seven days in greenhouse. Before planting, the soil was fertilized with NPK at the rate of 100, 109 and 137 µg.g-1 of soil, respectively, using NH_4NO_3 and KH_2PO_4 as the fertilizer sources. Twenty seeds of each variety were planted per pot (10 cm) and then thinned out to 15 plants soon after emergence. The nine tef varieties and the *E. curvula* var. Ermelo were planted into limed and unlimed soil, forming 20 treatment combinations. The experiment was set up in a randomized complete blocks design with 4 replications.

3.2.3 Data collection and analysis

Root and shoot length (mm) data were collected from each pot 30 days after planting from randomly selected plants and the mean of five plants was used for statistical analysis. Root and shoot dry weights (mg) were recorded on the basis of five randomly selected plants per replication after oven drying at $65^{O}C$ for 72 hours.

Tolerance indices (relative values) were computed as the ratio of the measured parameters under unlimed or toxic conditions, relative to the parameter measured under limed or nontoxic conditions. In addition, the shoot-to-root ratio was computed under both limed and unlimed conditions.

Analysis of variance and a single degree of freedom contrast, multiple means separation and correlation coefficients were carried out using GenStat Statistical Software Version:14 (GenStat., 2009).

3.3 Results

3.3.1 Genetic variability under unlimed treatments

Under unlimed conditions the acid soil (pH [KCl] 3.94 and acid saturation of 78%) caused variety specific responses for root length, shoot length, root dry weight, and shoot dry weight. The analysis of variance revealed highly significant differences

between the varieties ($p<0.01$) (Table 3.2). The single degree of freedom contrast showed a highly significant ($p<0.01$) difference between *E. curvula* var. Ermelo and all the *E. tef* varieties for all the parameters measured (Table 3.2). Contrast analysis between the pasture and food grain tef varieties did not show any significant differences for the parameters. On the other hand, comparison between brown seeded and white seeded tef varieties showed a highly significant difference ($P<0.01$) for all the parameters measured (Table 3.2). Stunted shoot growth coupled with severe root pruning effects were observed in the more sensitive varieties, which were the typical effects of Al-toxicity in the unlimed soil (Figures 3.2 and 3.3).

For all the parameters measured, the *E. curvula* variety showed better growth under unlimed conditions, followed by the brown seeded tef varieties Dima, Emmerson and SA Brown, in that order. Among the tef varieties, the highest and lowest values for mean root length and mean shoot length were recorded for Dima and Witkop varieties, respectively. Similarly, substantial variability was observed among the varieties for root dry weight and shoot dry weight (Table 3.3). Among the tef varieties, the lowest and highest root dry weights recorded were 3.38 mg and 10.45 mg for Highveld and Dima, respectively. For shoot dry weight Quncho and Highveld gave the smallest weights of 7.38 mg whereas the brown seeded tef variety, Dima produced 20.9 mg.

Table 3.1. Chemical properties of unlimed and limed sub-soil used for the study

Sample	Clay (%)	pH (KCl)	Na		K (mg. L^{-1})	Ca (mg. L^{-1})	Mg (mg. L^{-1})	Total Cation (Cmol. L^{-1})	Exc. acidity (Cmol.L^{-1})	Acid saturation (%)	P	Zn	Mn	Cu	Mid infrared estimate	
			mg/l	ESP (%)							mg. L^{-1}	mg. L^{-1}	mg. L^{-1} l	mg. L^{-1}	Organic Carbon (%)	N (%)
Unlimed	48	3.94	1.98	0.24	109	69	17	3.5	2.74	78	1	0.8	4	1.2	<0.5	0.07
Limed	47	6.21	3.08	0.17	119	1351	83	7.77	0.04	1	1	0.6	2	0.7	<0.5	0.05

ESP-Exchangeable sodium percentage

Table 3.2. Analysis of variance and orthogonal contrasts for growth parameters of nine tef varieties and *E. curvula* var. Ermelo grown in an unlimed, highly acidic soil[a].

Source of variation	d.f.		ARL	ASHL	RDWT	SHDWT
Block	3					
Varieties	9	P value	<0.001	<0.001	<0.001	<0.001
		F statistic	13.85	9.13	11.58	19.62
E. curvula vs *E .tef*	1	P value	<0.001	<0.001	<0.001	<0.001
		F statistic	83.51	38.56	67.03	120.56
Pasture vs. food grain varieties *(E. tef*)	1	P value	0.420	0.5	0.419	0.560
		F statistic	0.67	0.47	0.67	0.35
White vs. brown seeded varieties *(E. tef*)	1	P value	0.001	<0.001	0.008	<0.001
		F statistic	12.87	18.08	8.33	18.41
Residual	27					
Total	39					

[a]d.f-degrees of freedom; ARL-Mean root length; ASHL-Mean shoot length; RDWT-Root dry weight; SHDWT-Shoot dry weight.

Figure 3.1. Shoot growth of *E. tef* varieties and *E. curvula* var. Ermelo in limed and unlimed acid soils

Figure 3.2. Root length *E. tef* varieties and *E. curvula* var. Ermelo grown in a limed and unlimed acid soil

Table 3.3. Growth of tef varieties grown in an unlimed, highly acidic soil (pH=3.94)

Varieties	Seed colour	Use	ARL (mm)	ASHL (mm)	RDWT (mg)	SHDWT (mg)
Witkop	White	Pasture	15.45 a	8.85 a	4.90ab	8.50a
Quncho	White	Food	16.85 a	12.35ab	4.525ab	7.38a
Etsub	White	Food	18.60 a	12.25ab	4.50ab	8.28a
Rooiberg	Brown	Pasture	16.40 a	15.35bcd	5.30ab	10.10ab
Yilmana	White	Food	19.15 a	13.90bc	4.075a	7.85a
Highveld	Brown	Pasture	20.00 a	11.30ab	3.375a	7.38a
SA Brown	Brown	Pasture	25.35 ab	15.60bcd	5.550ab	11.35ab
Emmerson	Brown	Pasture	37.60 bc	18.50cd	7.55bc	14.80b
Dimma	Brown	Food	48.20 c	19.95de	10.45c	20.90c
E. curvula var. Ermelo	Brown	Pasture	72.20 d	24.10e	14.25d	30.45d
Mean			29.0	15.21	6.45	12.70
F statistic			13.85	9.13	11.58	19.62
P value			<0.001	<0.001	<0.001	<0.001
LSD (5%)			14.47**	4.38	2.92	4.95
CV (%)			34.4	19.8	31.2	26.8

[a]Means in the same column followed by the same letter are not significantly different at p=0.05.

[b]ARL-mean root length; ASHL-mean shoot length; RDWT-Root dry weight; SHDWT-Shoot dry weight

3.3.2 Variability for tolerance indices (relative values) and shoot to root ratio

Highly significant differences were observed for tolerance indices of all the growth parameters that were measured as ratio of the values under unlimed *versus* limed conditions. These indices indicated the extent of stress created by soil acidity relative to the limed or amended soils. Single degree of freedom contrasts between *E. curvula* and *E .tef* varieties showed a highly significant difference, which a large proportion of the variation between the varieties for all the tolerance indices. Similarly, there were highly significant differences between the brown and white seeded varieties of tef. However, there were no significant differences observed between pasture and grain varieties of tef for all the growth parameters (Table 3.4).

The tolerance indices for *E. curvula* ranged between 0.81 for root length to 0.98 for root dry weight. This reflects the extremely high tolerance of *E. curvula* variety to Al-toxicity and other stresses associated with the highly acidic soils. Lime had negligible effects on all the growth parameters measured for *E. curvula.* This result is consistent with earlier research reported on the species (Foy *et al.*, 1987; Miles and de Villiers,

1989; Foy and Murray, 1998). The tolerance indices or relative values among tef varieties ranged between 0.13-0.39 mm for relative root length; 0.23-0.43 mm for shoot length; 0.12-0.36 mg for root dry weight and 0.11-0.31 mg for shoot dry weight, indicating substantial variability between the tef varieties. Within tef varieties, the brown seeded grain variety Dima consistently gave the highest tolerance indices for all the growth parameters measured. The tef varieties generally responded more strongly to liming than *E. curvula*. However, severe suppression of growth of tef varieties under unlimed conditions resulted in very low tolerance indices of tef for all the parameters (Table 3.5).

Table 3.4. Analysis of variance and orthogonal contrasts of tolerance indices (relative values) of growth parameters under limed and unlimed conditions[a].

Source of variation	d.f.		RRL	RSHL	RRDWT	RSDWT
Block	3					
Varieties	9	P value	<.001	<.001	<.001	<.001
		F statistic	20.06	46.44	19.08	37.1
E. curvula vs *E. tef*	1	P value	<.001	<.001	<.001	<.001
		F statistic	151.72	380.23	158.73	317.74
Pasture vs food grain varieties *(E. tef)*	1	P value	0.04	0.201	0.932	0.453
		F statistic	0.85	1.72	0.01	0.580.
White vs brown seeded varieties *(E. tef)*	1	P value	0.002	0.001	0.031	0.041
		F statistic	12.47	12.89	5.2	4.58
Residual	27					
Total	39					

[a]RRL-Relative root length; RSHL-relative shoot length; RRDWT-Relative root dry weight; RSHDWT-Relative shoot dry weight.

Shoot to root ratio for the limed treatments gave highly significant differences and the values ranged between 2.7 for SA Brown to 1.95 for Yilmana. Under unlimed condition significant differences were not observed for shoot to root ratios. Generally, shoot to root ratios were reduced under unlimed condition (Table 3.5). A product-moment correlation coefficient indicated a high (>0.9) and highly significant correlation ($p<0.01$) between the growth parameters (Table 3.6). In screening experiments for Al-tolerance, shoot and root dry matter are usually recorded to capture variability in root density that cannot be accounted for by the length parameters *per se* (Miles and de Villiers, 1989; Liu, 2005). The high correlation between shoot and root length, and corresponding dry matter values observed in this experiment indicated the possibility that data recorded on length parameters can explain for root density.

Table 3.5. Tolerance ratios of growth parameters of tef varieties for root and shoot parameters[a,b]

Varieties	Seed colour	Use	RRL	RSHL	RRDWT	RSHDWT	SH:RT (DWT) Limed	SH:RT (DWT) unlimed
Witkop	White	Pasture	0.13a	0.21a	0.17a	0.12a	2.42abc	1.75
Quncho	White	Food	0.15ab	0.32bc	0.18ab	0.14a	2.10a	1.65
Etsub	White	Food	0.15ab	0.27ab	0.13a	0.12a	2.08a	1.86
Rooiberg	Brown	Pasture	0.16ab	0.31abc	0.22ab	0.15a	2.88	2.07
Yilmana	White	Food	0.16ab	0.31abc	0.12a	0.12a	1.95a	1.92
Highveld	Brown	Pasture	0.18ab	0.28ab	0.14a	0.12a	2.42abc	2.17
SA Brown	Brown	Pasture	0.27abc	0.33bc	0.25ab	0.20ab	2.78bc	2.02
Emmerson	Brown	Pasture	0.29bc	0.39cd	0.22ab	0.15a	2.82c	2.01
Dimma	Brown	Food	0.39c	0.44d	0.36b	0.31b	2.29abc	2.24
E. curvula var. Ermelo	Brown	Pasture	0.81d	0.93e	0.98c	0.96c	2.19ab	2.21
Mean			0.27	0.38	0.28	0.239	2.39	1.99
F statistics			20.06	46.44	19.08	37.10	3.21	0.61
P value			<.001	<.001	<.001	<.001	0.009	0.778
LSD (5%)			0.1344	0.0871	0.0836	0.1240	0.5402	NS
CV (%)			34.7	15.9	42.7	35.8	15.6	25.2

[a]Means in a column followed by the same letter are not significantly different at p=0.05. [b]RRL-Relative root length; RSHL-relative shoot length; RRDWT-Relative root dry weight; RSHDWT-Relative shoot dry weight; SH: RT- shoot to root ratio; DWT-dry weight

Table 3.6. Correlation coefficients between the various growth parameters measured in the study .

Parameter	ARL	ASHL	RDWT
ARL	-		
ASHL	0.9314**	-	
RDWT	0.9287**	0.9229**	-
SHDWT	0.9161**	0.9512**	0.9490**

ARL-mean root length; ASHL-mean shoot length; RDWT-Root dry weight; SHDWT-Shoot dry weight

3.4 Discussion

The primary effect of Al-toxicity is the inhibition of root growth, which eventually results in the reduced absorption of water and nutrients, and consequently the stunted growth of plants (Little, 1989; Delhaize and Ryan, 1995; Hede *et al.*, 2002; Deborah and Tesfaye, 2003; Kochian *et al.*, 2004; Miyasaka *et al.*, 2007). In this study, a high level of root pruning effects and stunted growth was observed among the tef varieties grown

under unlimed conditions. Root and shoot growth parameters of seedlings have been commonly used to evaluate genetic variability and to screen acid or Al-tolerant varieties in many crop and forage species (Little, 1989; Foy and Murray, 1998; Hede *et al.*, 2001; Liu, 2005; Dai *et al.*, 2011). In this experiment, high and statistically significant correlations were observed between all the parameters, indicating the appropriateness of these parameters for similar studies on tef. Under unlimed condition, the root and shoot growth of tef varieties were generally lower than those of the *E. curvula* variety, Ermelo. This can be attributed to the high tolerance of *E. curvula* to highly acidic soils (Foy *et al.*, 1987; Miles and de Villiers, 1989).

Similarly, the maximum tolerance index recorded for *E. tef* (0.44) for shoot length was very low compared to the values of over 0.9 recorded for *E. curvula*. As tolerance indices are the ratio of growth under unlimed (toxic) to limed (nontoxic) conditions, the low tolerance indices of tef varieties can be attributed to vigorous or weak growth of tef varieties under limed and unlimed condition, respectively. This can be seen clearly with the similar growth measurement of the Dima variety of tef and *E. curvula* under unlimed conditions (Table 3.2, Figs 3.1 and 3.2).

Assefa *et al.* (2010) described the existing tef cultivar development strategy as breeding for general adaptation. However, the *E. tef* varieties tested in this study had not been bred for tolerance to acid soils or Al-tolerance. Recent figures on variety development in tef have shown a decline in genetic gain, mainly because of a lack of specifically adapted varieties and the presence of strong genotype by environment interactions (Assefa *et al.*, 2010).The large differences in the responses of nine tef varieties to an acid soil reflect the need to launch tef breeding programmes specifically targeting such agro-ecologies.

3.5 Conclusion

The highly acid subsoil used for the experiment was effective at exposing the intraspecific genetic variation in tef tolerance for Al-toxicity and other acidity associated stresses. The tef varieties used in this experiment were not intentionally bred for Al-tolerance and the considerable variation observed suggests the possibility of selecting tef varieties with high level of tolerance to Al-toxicity among diverse tef accessions. In this regard, deliberate screening of tef accessions collected from areas with acid soils could be an effective starting point. A consistent association of brown seed colour with

tolerance to Al toxicity in this experiment suggests that further research might show a genetic linkage between brown seed colour and tolerance of acid soils.

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CHAPTER 4

Responses of selected tef [*Eragrostis tef* (Zucc.) Trotter] genotypes to soil acidity in pot and field experiments

Abstract

Soil acidity causes substantial yield losses on tef production in Ethiopia. There has been no systematic investigation conducted on the response of tef genotypes to soil acidity and Al-toxicity. The objectives of this study was to examine the response of 31 tef genotypes together with two related *Eragrostis* spp., and to assess farmers' preference and selection criteria for tef genotypes. The tef genotypes were constituted from improved varieties, parents of mapping populations, and a local check. Growth measurements and tolerance indices were used to evaluate the responses of the genotypes. There were significant difference between the genotypes under both the pot and field conditions. Significant correlations were observed between tolerance indices under both conditions. Nonetheless, within and between variations in ranks were observed for the tolerance indices for the pot and field experiments. Within experiment changes in rank were associated with the inherent properties of the tolerance indices and the parameter used. The rank changes between pot and field experiments could be attributed to the difference in adaptability of some genotypes to other edaphic and climatic factors in the test conditions. The local check, consistently outperformed the other genotypes, both under pot and field conditions. A lack of adequate contrast between the three parents of the mapping population rules out the possibility of using the two mapping populations developed from these parents for molecular mapping of tolerance to soil acidity and Al-toxicity. Grain yields of the 'improved' varieties, as well as the local check under unlimed condition, were far below the national mean yield of tef. This highlights the need to develop tef varieties that are tolerant to acid soils; agronomically superior and can perform adequately in agro-ecologies with acid soils. Farmers selected one late maturing variety and three early maturing varieties to grow in their moderately acidic soils.

Key words: Aluminium toxicity, *Eragrostis tef*, soil acidity, tolerance indices

4.1. Introduction

Tef [*Eragrostis tef* (Zucc.) Trotter] (2n=4x=40) is the most widely produced and consumed cereal crop in Ethiopia. In terms of area of cultivation, it is the leading cereal crop followed by maize and wheat. According to the Central Statistical Authority of Ethiopia (CSA, 2015), the area covered by tef during the 20014/2015 cropping season was over 3 million hectares corresponding to 24.03% and 30% of the total area occupied by grain and cereal crops, respectively. In the country, over 6 million rural households are engaged in tef production (CSA, 2015). Besides the grain, tef straw is also highly valued as an important livestock feed in the country. Tef straw contributes 27% of the total of 14 million tons of crop residue produced in the country (Seyoum and Dereje, 2001). Tef is gaining growing popularity worldwide primarily due to its gluten free property (Spaenij-Dekking *et al.*, 2005).

Aluminium toxicity and other acidity related soil fertility problems are among the major constraints of crop production in the Ethiopia, affecting most crops (Dubale, 2001; IFPRI, 2010). Tef is one of the major crops affected by soil acidity and Al-toxicity. Mamo and Killham (1987) reported the poor response of tef to fertilizer applications when grown in acid soils. Nonetheless, breeding of tef for specific adaptation to low soil pH or aluminium toxicity has not yet been initiated. The national tef improvement programme of Ethiopia Agricultural Research system has released several high yielding varieties. However, most of these varieties have been bred for optimal growing conditions, with a few of them being specifically bred for drought tolerance (Assefa *et al.*, 2010). High levels of genotype by environment interaction have been reported in several tef breeding trials (Kassa *et al.*, 2006; Assefa *et al.*, 2010; Ashamo and Getachew, 2012). Declining overall genetic gain from the national breeding programme has been associated with the failure in the breeding approaches to target specific production constraints (Assefa *et al.*, 2010). Cool temperatures at high altitudes and moisture stress in drought prone areas have been implicated as the underlying factors causing low productivity and the high genotype by environment interactions (Assefa *et al.*, 2010). Although soil acidity is an edaphic factor constraining crop productivity in the ‘optimal growing’ environments, its role in the high genotype by environment interactions and the overall declining genetic gain has not been recognized widely. A popular and supposedly well adapted tef variety, *Quncho*, (Assefa *et al.*, 2011) has been found to be among the most Al-sensitive tef varieties

(Abate *et al.*, 2013). Overall, there is little information on the reaction of most tef varieties to soil acidity and the associated Al-toxicity.

Crop genotypes can be screened for Al-toxicity or soil acidity under field conditions or in controlled environment facilities. Compared to field-based techniques, controlled environment methods under laboratory and greenhouse conditions can be more rapid, accurate and non-destructive, and can be applied at early developmental stages of the crop (Howeler and Cadavid, 1976; Carver and Ownby, 1995; Hede *et al.*, 2001). The ultimate purpose of screening in a controlled environment is the rapid and large scale screening of many promising genotypes before confirmatory field evaluations and subsequent seed production of the best lines. Hence, the evaluation of selected genotypes for yield and other economically important traits under field condition is essential (Hede *et al.*, 2001). Field evaluations help to assess the response of genotypes under the influence of complex edaphic and climatic factors, in addition to low soil pH. Furthermore, field experiments can be used to gather farmers' feedback towards the genotypes.

Under controlled environmental conditions, the relative growth index of seedlings, expressed as the ratio of growth under unlimed condition to limed conditions, has been widely used to identify tolerant lines (Bona *et al.*, 1993; Hede *et al.*, 2001; Liu, 2005). Tolerance indices are also used to screen crops for tolerance of other abiotic stresses such as drought (Shirani and Abbasian, 2011; Khalili *et al.*, 2012; Abdi *et al.*, 2013; Abdolshahi *et al.*, 2013).

This study was conducted in order to evaluate the response of 31 tef genotypes to Al-toxicity or soil acidity in pot and field experiments, and to assess farmers' preferences and selection criteria.

4.2 Material and methods

4.2.1 Greenhouse experiment

4.2.1.1 Genetic stock

Thirty one (*E .tef*) genotypes consisted of 28 Released Varieties, 2 parents of mapping population (*Key murrie,* and DZ-01-2785) and a farmers' landrace called *Dabo banja* were evaluated, together with two *Eragrostis* spp. (*Eragrostis pilosa* (L.) Beauv. and *Eragrostis curvula* (Schrad.) Nees var. Ermelo) under greenhouse conditions at the

Amhara Regional Agricultural Research Institute (ARARI), Bahir Dar, Ethiopia (Table 4.1). *Key Murrie*, DZ-01-2785 and *E. pilosa* (Acc 30-5) were used to develop two sets [*E. tef* (*Key Murrie*) X *E. pilosa* (30–5)] and [*E. tef* (DZ-01-2785) X *E. pilosa* (30–5)] of recombinant inbred lines to be used as mapping populations for a molecular mapping studies of tef (Solomon, 2007; Assefa *et al.*, 2010). *Eragrostis curvula* var. Ermelo was used as an Al-tolerant check (Abate *et al.*, 2013). *Dabo banja* was identified as a widely grown farmers' landrace in areas of *Banja* district with acid soils during the PRA Study.

4.2.1.2 Experimental set up

A sample of an acidic soil with a pH (H_2O) $_{1:2.5}$ and pH (KCl) of 4.45 and 3.68 was collected from the *Banja* District of north western Ethiopia. The soil was analysed for various physico-chemical properties at the Amhara Design and Supervision Works Enterprise, Soil Chemistry and Water Quality Section, Bahir Dar, Ethiopia (Table 4.2).

In order to facilitate computation of tolerance indices, the experiment was established under limed and unlimed conditions. Accordingly, the acid soil was limed to a pH of 6.2 by applying 8.5 g of $CaCO_3$ (99.5%) powder per kilogram of dry soil (17 t lime ha^{-1}) (Nyachiro and Briggs, 1988) and incubating the limed soil for seven days in a greenhouse. Before planting, the soil was fertilized with NPK at the rate of 100, 109 and 137 µg.g-1 of soil, respectively, using NH_4NO_3 and KH_2PO_4 fertilizers. Seeds were planted in 10 cm pots. All the varieties were planted in limed and unlimed pairs. The experiment was set up in in a randomized complete blocks design (RCBD), with 5 replications.

4.2.1.3 Data collection

Shoot and root length (mm) data were collected from each pot 28 days after planting from randomly selected plants, and the mean of 7 plants was used for statistical analysis. Root and shoot dry weights (mg) were recorded on the basis of 10 randomly selected plants per replication after oven drying at $65^{O}C$ for 72 hours.

Tolerance indices (relative values) were computed as the ratio of the measured parameters under unlimed *versus* limed conditions.

4.2.2 Field experiment

4.2.2.1 Genetic stock

Twenty seven (Table 4.1) improved varieties of tef, along with popular local farmers' landrace, *Dabo banja*, were used in this trial.

4.2.2.2 Experimental set up

A field experiments was conducted at a newly established experimental station in the *Banja* district of north western Ethiopia during the main rainy season of 2014. *Banja* area represents the most acidic, high rainfall highlands of north western Ethiopia. A detailed description of the district is presented in Chapter 2. The soils of the testing site was analysed for soil pH and selected physico-chemical properties by collecting a composite sample of the field at a depth of 15 cm and following appropriate lab protocols described under Chapter 2. The analysis was carried out at the soil analysis laboratory of the Adet Agricultural Research Centre. The experiment was replicated in farmers' field in order to gather data on farmers' preferences and selection criteria.

The experiment was laid out in a randomized complete blocks design (RCBD) with two replications with limed and unlimed treatment pairs. Liming rates of 8.0 $t.ha^{-1}$ and 6.0 $t.ha^{-1}$ were used for the on-station and on-farm experiments, respectively, based on the soil test result (Table 4.2). The lime was applied two weeks before planting the limed plots.

Each genotype was established in a plot with the area of 1.0 m^2, with an inter row spacing of 20 cm. Seeds were drilled within rows and seed rate of 15 $kg.ha^{-1}$ was used. Spacings of 1.0 m between plots within replications and 1.5 m between blocks were used. Fertilizers were applied based on the recommendation under use in the area, i.e.,100 $kg.ha^{-1}$ DAP and 100 $kg.ha^{-1}$ urea. All of the DAP and one third of the urea was applied at planting, and the remaining two thirds of the urea was applied at the tillering stage.

4.2.2.3 Data collection

The following data were collected on a plot basis or from randomly pre-tagged individual plants in the central rows.

Data collected on a plot basis:

- *Days to panicle emergence (DPE)*: Number of days from planting until emergence of panicles on 50% of the plants in the plots;
- *Days to maturity (DM)*: Number of days from planting to the day when 50% of the plants in the plot reached physiological maturity;
- *Shoot biomass (SB):* Total dry above-ground biomass for the entire plot;
- *Grain yield (GY):* The weight of the seed harvested from each plot;
- *Establishment*: Rated 30 days after planting and at harvest, with the following visual scale of 1-5:
  5-(>85% establishment)
  4-(70-84 establishment)
  3-(55-69% establishment)
  2-(40-54% establishment)
  1-(below 40% establishment)

Data was collected from five randomly selected and pre-tagged plants basis:

- *Plant height (PH):* mean height of 5 pre-tagged plants in centimetres
- *Panicle length (PNL):* mean length of 5 pre-tagged plants from the base of the panicle to the tip in centimetres.
- *Tiller number (TN):* the mean number of fertile tillers of 5 pre-tagged plants in each plot
- *Weight of panicle branches per main shoot panicle (NPBPPN):* the mean dry weight in grams of the above-ground biomass of 5 pre-tagged plants in each plot.

Computation of acidity tolerance indices: these indices were computed as follows:

i. *Relative tolerance index (RTI)-STI-1:* Ratio of values under unlimed or stressed *versus* the values under limed or non-stressed conditions;

$$STI\text{-}1 = Y_s/Y_p$$

ii. *Stress tolerance index-2 (STI-2)* (Fernandez, 1992)

$$STI\text{-}2 = (Y_s)\,(Y_p)/\,(\bar{Y}_p)^2$$

iii. *Stress tolerance index*-3 (STI-3) modified from STI-2 of (Fernandez, 1992)

$$STI\text{-}3 = Y_s/\,\bar{Y}_p$$

iv. *Stress tolerance index-4 (STI-4)* (Fischer *et al.*, 1998)

$$STI\text{-}4 = (Y_s/Y_p)/(\bar{Y}_s/\bar{Y}_p)$$

Where $\mathbf{Y_s}$ is yield or any other variable for each genotype under stress or unlimed condition; $\mathbf{Y_p}$ is the same variable under non-stressed or limed condition; $\mathbf{\bar{Y}_s}$ is the grand mean of yield or any other variable of all genotypes under stress; $\bar{Y}_p$ is grand mean of the same variable for all genotypes under non stressed condition.

Farmers' assessment of varieties: Twenty- five farmers in two groups of 12 and 13 assessed the varieties under unlimed condition for various attributes on the 3rd of November 2014: The landraces *Dabo banja* and *Feso* served as benchmarks for the farmers to use as the basis for assessment. *Dabo banja* is a widely cultivated and relatively late maturing landrace, whereas *Feso* is an early maturing landrace used in double cropping of tef after potato. Farmers' preferred attributes and selection criteria were documented.

4.2.3 Statistical analysis

Measurements of each parameter and their associated tolerance indices were subjected to analysis of variance and means separation. Hierarchical cluster analysis was undertaken to visualize the grouping of the genotypes using their similarities based on growth values under unlimed condition and their tolerance indices, independently. Euclidean distances between the genotypes was used to group the genotypes. Correlation analyses between tolerance indices and growth parameters were also performed in order to determine their pattern of association. Grain and biomass yield under unlimed conditions and tolerance indices computed from these variables were used for statistical analysis of the field experiment. Analysis of variance, mean separation and correlation analysis were also performed. GenStat Statistical Software Version:17.10013780 (GenStat., 2014) was used to undertake all the statistical analysis.

Table 4.1. Description of tef genotypes used for the study.

No.	Name	Code/Pedigree	Released from (centre)	Year of Release	Seed colour	Test Environment
1	*Amarech*	Ho-Cr-136	Holleta	2006	White	P/F
2	*Ambotoke*	DZ-01-1278	Holleta	2000	White	P/F
3	*Asorgi*	DZ-01-99	Debrezeit	1970	Brown	P/F
4	*Boset*	RIL 50D	Debrezeit	2012	White	P
5	*Dega Tef*	DZ-01-2675	Debrezeit	2005	White	P/F
6	*Dimma*	DZ-01-2423	Adet	2005	Brown	P/F
7	*Dukem*	DZ-01-974	Debrezeit	1995	White	P/F
8	*Enatit*	DZ-01-354	Debrezeit	1970	White	P/F
9	*Etsub*	DZ-01-3186	Adet	2005	White	P/F
10	*Gemechis*	DZ-Cr-387/RIL-127	Melkassa	2007	White	P/F
11	*Genete*	DZ-01-146	Sirinka	2005	White	P/F
12	*Gerado*	DZ-01-1281	Sirinka	2002	White	P/F
13	*Gibe*	DZ-01-255	Debrezeit	1983	White	P/F
14	*Gimbichu*	DZ-01-899	Debrezeit	2005	White	P/F
15	*Holeta Key*	DZ-01-2053	Holleta	1999	Brown	P/F
16	*Keytena*	DZ-01-1681	Debrezeit	2002	Brown	P/F
17	*Koye*	DZ-01-1285	Debrezeit	2002	White	P/F
18	*Magna*	DZ-01-196	Debrezeit	1978	White	P/F
19	*Mechare*	Acc.205953	Sirinka	2007	White	P/F
20	*Melko*	DZ-Cr-82	Debrezeit	1982	White	P/F
21	*Menagesha*	DZ-01-44	Debrezeit	1982	White	P/F
22	*Quncho*	DZ-Cr-387/RIL-355	Debrezeit	2006	White	P/F
23	*Simada*	DZ-Cr-387/RIL-295	Debrezeit	2009	White	P/F
24	*Tseday*	DZ-Cr-37	Debrezeit	1984	White	P/F
25	*Welenkomi*	DZ-01-787	Debrezeit	1978	White	P/F
26	*Yilmana*	DZ-01-1868	Adet	2008	White	P/F
27	*Ziquala*	DZ-Cr-358	Debrezeit	1995	White	P/F
28	*Zobel*	DZ-01-1821	Sirinka	2005	White	P/F
29	*Dabo banja*	Local check			Brown	P/F
30	DZ-01-2785	PMP			white	P
31	Kay Murrie	PMP			white	P
32	*E. pilosa* (Acc 30-5)	PMP			Brown	P
33	*E.curvula* var. Ermelo	Tolerant check			Brown	P
P-pot; P/F-pot and field; PMP- parents of mapping population						

Table 4.2. pH and other physico-chemical properties of the soil used for the pot experiment and in the field

No	Experiment	Lime Treatment	pH H_2O) 1:2.5	pH (KCl)	Exchangeable bases (Cmol(+).kg^{-1}				CEC	N total (%)	Av.P	Ex.Ac.	Ex.Al
					Ca	Mg	Na	K	(Cmol(+).kg^{-1}		(mg.kg^{-1})	(Cmol(+).kg^{-1}	
1	Pot	Limed	6.23	5.48	46.75	0.05	0.01	0.61	22.00	0.478	5.75	5.68	0
		Unlimed	4.45	3.68	13.03	0.12	0.12	0.56	23.40	0.384	5.33	18.64	4.16
2	Field: On farm	Limed	6.2	5.07	18.90	0.77	0.04	0.06	38.20	0.6	25	0.058	0
		Unlimed	4.89	3.8	0.29	1.75	0.02	0.06	32.88	0.56	29.14	4.14	3.57
3	Field On station	Limed	6.13	4.87	14.50	1.56	0.00	0.07	27.85	0.41	2.02	1.14	0.05
		Unlimed	4.42	3.75	0.16	2.14	0.01	0.07	30.36	0.33	1.21	5.13	5.05

Av. P- available phosphorous; CEC- Cation exchange capacity; Ex Ac.- exchangeable acidity; Ex Al.-exchangeable Al

4.3 Results

4.3.1 Response of tef genotypes to soil acidity in the pot experiment

The response of the tef genotypes to soil acidity in terms of their growth parameters under unlimed conditions and their relative tolerance indices are presented in Tables 4.3 and 4.4, respectively. There were significant differential responses by the varieties to soil acidity, suggesting that the test soil used in the experiment had adequately discriminated between the genotypes.

Based on the measurement of growth parameters under unlimed condition, the root dry weight (RDW), mean root length (ARL), mean shoot length (ASHL), and shoot dry weight (SHDW) values of the best performing varieties were 3.4, 2.95, 1.69 and 2.1 times higher than the worst performing varieties, respectively. The tolerant check *E. curvula* var. Ermelo ranked 27th for RDW and ARL, and 30th and 18th for SHL and SHDWT, respectively. This could be attributed to the initial slow growth of this perennial species compared to the tef genotypes. The local check, *Dabo banja*, which is widely grown in areas with acid soils, ranked 2nd, 5th, 1st for RDWT, ARL and SHL and SHDWT, respectively.

The result of the relative tolerance index (RTI) confirmed the significant superiority of the local check, *Dabo banja*, over all the improved varieties, the parents of the mapping populations and the tolerant check, *E. curvula* var. Ermelo. Figure 4.1 shows the contrast between the local check (*Dabo banja*) and the most sensitive variety (*Holleta Key*) and the most popular variety (*Quncho*) (Belay *et al.*, 2008; Assefa *et al.*, 2011) under limed and unlimed conditions. The RTI values derived from roots and shoots were over 100% for the local check. The RTI also showed the high tolerance of *E. curvula* var. Ermelo, which was not apparent from the use of growth measurements as indicators of tolerance to soil acidity. All three parents of the mapping populations, i.e., *Key Murrie*, DZ-01-2785 and *E. pilosa* (Acc 30-5) showed poor tolerance to soil acidity for their growth parameters, and their soil acidity tolerance indices.

The differences observed for both the growth and RTI values presents an opportunity to select for relatively acid tolerant varieties among the existing varieties.

Table 4.3. Root and shoot growth of tef genotypes grown in unlimed soils

No	Variety	RDWT (mg)	Rank	ARL (mm)	Rank	ASHL (mm)	Rank	SHDWT (mg)	Rank
1	*Mechare*	25.22	1	87.49	3	97.03	16	38.83	4
2	*Dabo banja*	24.40	2	85.54	5	117.91	1	56.83	1
3	*Enatit*	24.40	3	89.20	2	95.11	18	34.17	12
4	*Genete*	23.60	4	83.83	8	102.26	9	36.83	6
5	*Gerado*	23.60	5	82.43	10	99.89	10	31.83	16
6	*Ziquala*	23.40	6	97.46	1	104.43	6	38.83	4
7	*Menagesha*	23.20	7	82.11	11	98.69	13	47.17	2
8	*Gibe*	22.45	8	83.09	9	106.40	5	36.17	8
9	*Asorgi*	22.00	9	85.34	6	107.86	3	38.83	4
10	*Dimma*	21.00	10	86.08	4	106.57	4	36.50	7
11	*Tseday*	20.80	11	70.74	19	103.5	7	37.83	5
12	*Amarach*	20.20	12	71.76	18	107.97	2	41.50	3
13	*Yilmana*	19.80	13	70.20	20	99.63	11	33.17	14
14	*Gimbichu*	19.60	14	72.13	17	93.43	19	28.17	20
15	*Boset*	19.20	15	68.46	21	103.43	8	32.17	15
16	*Zobel*	19.20	16	73.60	15	91.86	20	34.50	11
17	*Melko*	19.00	17	84.43	7	95.91	17	31.83	16
18	*Magna*	18.80	18	64.17	24	85.14	24	27.83	21
19	*Ambotoke*	17.80	19	75.74	14	98.89	12	35.50	9
20	*Koye*	17.80	20	72.51	16	89.94	22	25.50	23
21	*Keytena*	17.40	21	77.24	12	98.14	14	35.17	10
22	*Welenkomi*	17.20	22	60.57	28	78.03	29	23.17	24
23	*Etsub*	17.00	23	76.51	13	86.80	23	33.83	13
24	DZ-01-2785	16.80	24	67.17	22	97.83	15	38.17	5
25	*Dega Tef*	16.20	25	65.14	23	90.89	21	31.50	17
26	*Dukem*	15.80	26	64.00	25	80.51	26	26.17	22
27	*E. curvula*	15.40	27	60.86	27	77.14	30	29.50	18
28	*Gemechis*	15.40	28	62.31	26	80.37	27	22.50	25
29	*Simada*	15.20	29	57.60	29	82.40	25	19.50	26
30	*Quncho*	13.40	30	49.63	31	78.29	28	19.50	26
31	*Kay Murrie*	11.20	31	57.17	30	71.00	32	34.50	12
32	*E. pilosa*	10.00	32	43.51	32	76.74	31	28.83	19
33	*Holeta Key*	7.40	33	31.73	33	70.26	33	18.17	27
	Mean	**18.6**		**71.5**		**92.93**		**32.86**	
	F static	**4.02**		**5**		**4.48**		**15.09**	
	P value	**<0.001**		**<0.001**		**<0.001**		**<0.001**	
	LSD (5%)	**5.97**		**17.62**		**15.68**		**5.73**	
	CV (%)	**25.7**		**19.7**		**13.9**		**13.5**	

ARL-Mean root length; ASHL-Mean shoot length; RDWT-Root dry weight; SHDWT-Shoot dry weight

There were highly significant correlations (P<0.001) between all growth parameters and all the RTI values, with the exceptions of RSHL (relative shoot dry weight) and SHL, RSHL and RDW, and SHL and RRDW (Relative Root Dry Weight) (Table 4.5).

There was considerable change in rank of the varieties for their growth parameters and their relative tolerance indices. Visualization of the genotypes through clustering showed differential grouping of the genotypes for RTI and growth parameters under unlimed condition (Figures 4.2 and 4.3). However, the RTI data grouped together the

two genotypes with proven acidity tolerance, i.e., the local check from acid soils and *E. curvula*.

Table 4.4. Relative soil acidity tolerance indices of tef varieties derived from shoot and root growth

No	Variety	RRL(%)	Rank	RRDW (%)	Rank	RSHL (%)	Rank	RSHDWT (%)	Rank
1	Dabo banja	108.26	1	128.2	1	121.85	1	149.46	1
2	*E.curvula*	102.21	2	115.11	2	106.67	2	133.56	2
3	Ziquala	101.51	3	76.00	10	78.68	10	71.00	10
4	Dimma	96.43	4	85.46	3	94.02	3	88.56	3
5	Melko	93.75	5	63.50	21	76.63	13	62.51	19
6	Genete	91.9	6	80.85	5	85.30	4	73.37	5
7	Asorgi	88.02	7	68.02	14	78.44	11	64.95	16
8	Keytena	87.79	8	67.64	18	74.37	15	66.92	14
9	Gibe	85.59	9	79.45	6	83.84	6	69.04	12
10	Mechare	85.41	10	85.23	4	76.07	14	71.91	7
11	Ambotoke	85.01	11	43.28	29	62.78	29	59.46	22
12	Enatit	83.78	12	68.06	13	72.52	18	59.19	23
13	Gerado	81.87	13	78.04	8	84.73	5	71.03	9
14	Tseday	81.36	14	79.32	7	77.25	12	70.12	11
15	Gimbichu	81.26	15	74.88	11	80.09	8	51.81	26
16	Magna	80.06	16	61.62	24	66.09	26	61.95	20
17	Dukem	79.47	17	52.83	26	70.95	20	46.69	28
18	Amarach	79.21	18	77.23	9	68.65	22	53.85	25
19	Zobel	79.18	19	67.97	15	73.97	17	67.17	13
20	Menagesha	77.27	20	72.60	12	65.71	28	72.55	6
21	DZ-01-2785	77.15	21	67.58	17	81.58	7	71.68	8
22	*Dega Tef*	76.91	22	63.67	20	72.32	19	63.20	18
23	*Koye*	74.45	23	52.72	27	66.13	25	41.75	31
24	*Boset*	70.31	24	64.39	19	79.32	9	64.83	17
25	*Simada*	66.87	25	61.79	23	54.94	32	42.55	30
26	*Quncho*	64.33	26	49.89	27	65.56	27	44.51	29
27	*Yilmana*	64.24	27	53.35	25	74.23	16	65.53	15
28	*Etsub*	63.21	28	42.12	30	68.93	21	60.84	21
29	*Welenkomi*	60.35	29	62.10	22	66.71	24	49.49	27
30	*Gemechis*	58.08	30	38.04	32	55.31	31	41.01	32
31	*Kay Murrie*	54.22	31	43.54	28	68.42	23	73.83	4
32	*Holeta Key*	39.30	32	27.51	33	50.56	33	34.02	33
33	*E. pilosa*	39.20	33	38.94	31	62.04	30	54.34	24
	Mean	**77.51**		**66.4**		**74.69**		**65.84**	
	F static	**11.6**		**10.88**		**7.48**		**23.58**	
	P value	**<.001**		**<.001**		**<.001**		**<.001**	
	LSD (5%)	**13.261**		**17.45**		**14.255**		**13.232**	
	CV (%)	**13.7**		**21**		**15.3**		**16.1**	

RRL-Relative Root Length; RRDW-Relative Root Dry Weight; RSHL-Relative Shoot Length; RSHDWT-Relative Shoot Dry Weight

Figure 4.1. Growth of sensitive and tolerant tef varieties in unlimed and limed soil from the Banja District, Ethiopia (pH(KCl) 3.68)

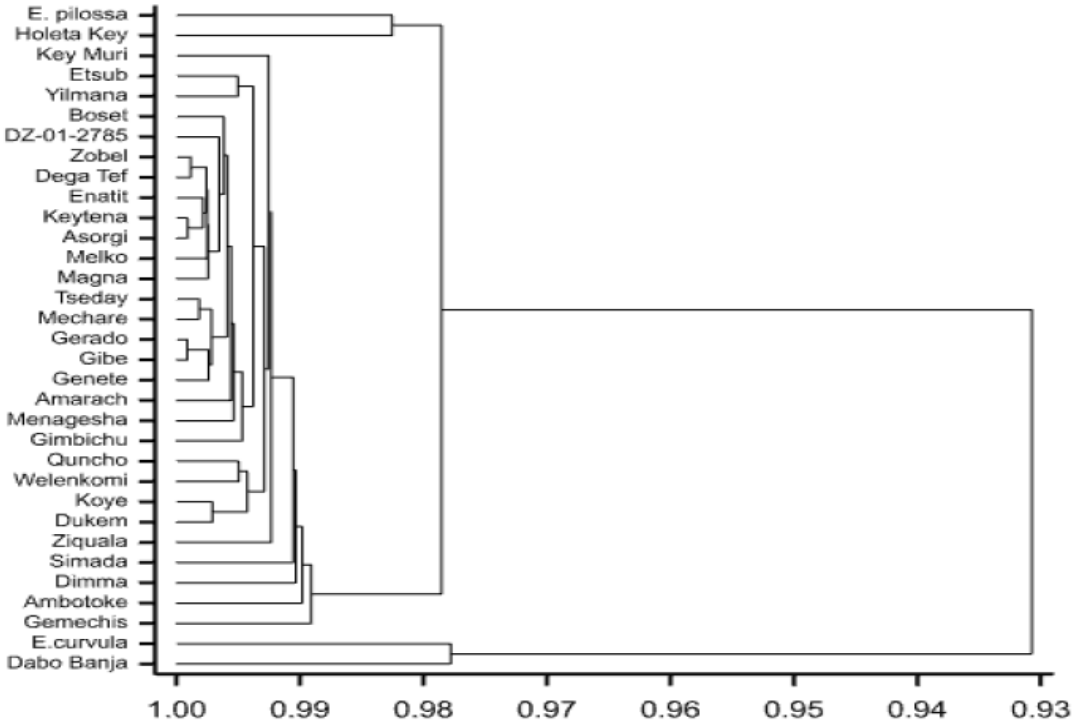

Figure 4.2 Clustering of tef genotypes based on soil acidity reaction; similarities are indexed by relative tolerance indices

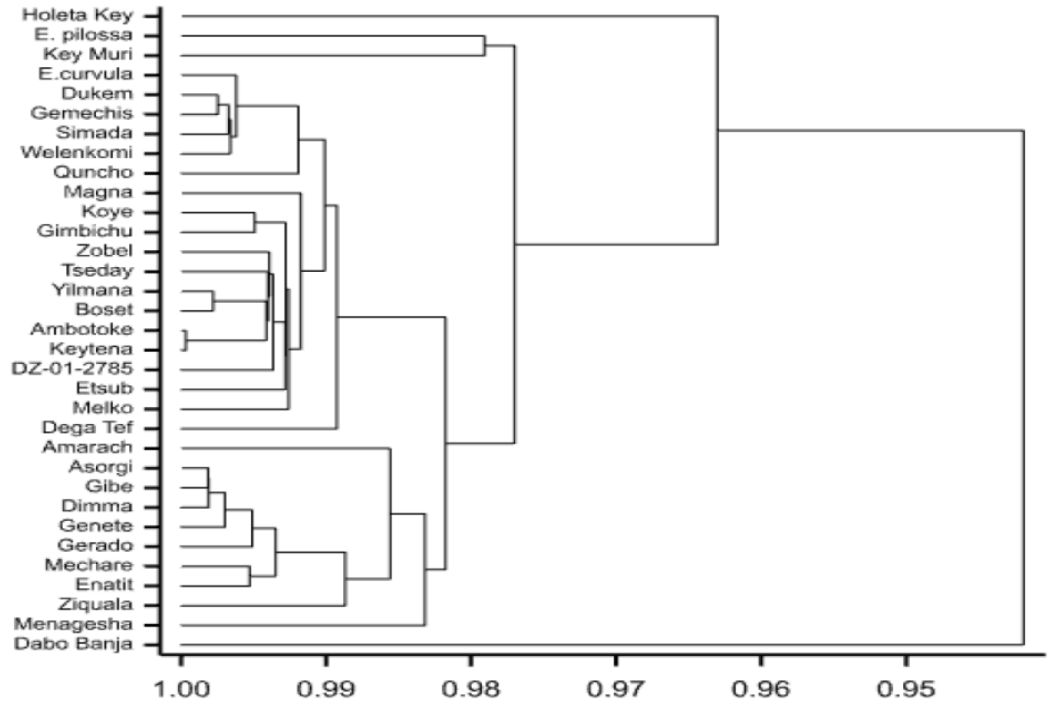

Figure 4.2 Clustering of tef genotypes based on soil acidity reactions; similarities indexed by growth under unlimed condition.

Table 4.5. Correlation analysis of root and shoot growth measurements (unlimed) and relative acidity tolerance indices of tef genotypes (n=165)

RDW	1	-							
RRDW	2	0.44***	-						
RRL	3	0.46***	0.68***	-					
RSHDW	4	0.26***	0.71***	0.52***	-				
RSHL	5	0.17NS	0.74***	0.56***	0.72***	-			
ARL	6	0.75***	0.40***	0.60***	0.28***	0.24**	-		
SHDW	7	0.50***	0.51***	0.45***	0.64***	0.47***	0.54***	-	
ASHL	8	0.74***	0.16NS	0.28***	0.21**	0.06NS	0.55***	0.46***	-
		1	2	3	4	5	6	7	8
		RDW	**RRDW**	**RRL**	**RSHDWT**	**RSHL**	**RL**	**SHDWT**	**SHL**

***= P<0.001; **= P<0.01; *= p<0.05; NS= not significantly different at P=0.05; RDW=root dry weight; RRDW=relative root dry weight; RRL=relative root length; RSHW=relative shoot dry weight; RSHL-relative shoot length; ARL=mean root length; SHDW=shoot dry weight; ASHL=mean shoot length.

4.3.2 Response of tef varieties to soil acidity in field experiments

The combined ANOVA showed highly significant differences (P<0.001) between the genotypes for grain yield under unlimed condition and for all the acidity tolerance indices. Similarly, a significant difference was observed between the two sites for grain yield under unlimed condition (Table 4.6). The mean grain yield of unlimed on-farm plots (0.814 t.ha-1) was higher than the mean grain yield from on-station plots (0.383 t.ha-1) (Table 4.7). The soil tests showed that the on-farm plots were less acidic than the on-station plots (Table 4.2). None of the other tolerance indices showed significant differences between the two sites. Significant differences were observed for genotype by site interaction only for STI-2 (P<0.001). STI-2 tended to select for genotypes with better performances on limed plots.

The local check, *Dabo banja*, consistently ranked among the top two genotypes across all the tolerance indices and testing sites. It was notably superior in the most acidic of soils, found in the more discriminating on-station environment. Nevertheless, its grain yield at both sites was below the national mean for tef (1.58 t.ha-1) (CSA, 2015). Among the improved varieties, *Gibe* and *Tsedey* consistently gave better yields across the test sites, and registered good tolerance indices. But the overall yields of these varieties were poor compared to the current national mean productivity of tef. The tolerance indices showed variation in their ability to identify sensitive varieties. *Holleta Key, Magna, Dukem, Yilmana, Menagesha, Quncho* were among the most sensitive varieties across all the acidity tolerance indices (Table 4.7).

The yields of above-ground shoot biomass of genotypes also showed highly significant differences (P<0.001) across all the tolerance indices and the test sites (Table 4.8). The two locations showed significant differences (P<0.001) for all the tolerance indices, except for STI-4 (P=0.604). Variety by site interactions were only identified for STI-2 (Table 4.8). As with the grain yield, the overall mean of above-ground biomass was higher for the on-farm site than on station, i.e., 3.44 $t.ha^{-1}$ vs 2.42 $t.ha^{-1}$ (Table 4.9). The local check, *Dabo banja*, consistently gave the highest above-ground biomass yields followed by the improved variety *Gibe*. The tolerance indices with significant variety-by-site interactions resulted in rank changes between sites, specifically in identification of the most sensitive varieties. STI-1 and STI-4 computed from grain yield and above-ground biomass gave similar results for variety, site, and variety- by-site interactions. On the other hand, the results for STI-2 and its modified version, STI-3, used in this study differed considerably, suggesting the possibility of their complementary use.

Correlation analysis among tolerance indices showed a weak association of yield of limed plots with most of the tolerance indices (Table 4.10). But highly significant associations were observed between limed plots and unlimed plots for both grain yield and above-ground biomass. Grain and above-ground biomass yields from unlimed plots, on the other hand, strongly associated with all the tolerance indices, reflecting the importance of using actual performance of genotypes under stressed conditions for the selection of tolerant genotypes. STI-I and STI-4 showed perfect association (r=1, P<0.001) for grain yield and (r=0.9, P<0.001) for above-ground biomass (Table 4.10). STI-4 is normally STI-1 divided by the same denominator, i.e., the mean unlimed value divided by the mean limed value. Consequently, STI-4 was removed from subsequent analysis.

Table 4.6. Summary of combined analysis of variance for soil acidity tolerance indices of grain yield under on-station and on-farm experiments

Source of variation	Df		UL GY	STI-1	STI-2	STI-3	STI-4
Block	1						
Variety	27	F-statistic	3.92	6.06	12.91	9.91	6.05
		P-value	<.001	<.001	<.001	<.001	<.001
Site	1	F-statistic	34.99	0	0.8	0.02	0.05
		P-value	<.001	0.96	0.376	0.876	0.832
Variety. Site	27	F-statistic	1.32	0.57	5.25	2.17	0.57
		P-value	0.191	0.941	<.001	0.008	0.941
Residual	55						
Total	111						

UL GY-Unlimed grain yield STI-1-4- Stress tolerance indices 1-4 , as described in section 4.2.2.2

Table 4.7. Acidity tolerance indices of tef varieties computed from their above-ground biomass

Varieties	GY-UL (T.ha^{-1})				STI-1		STI-2				STI-3				STI-4	
	(OS)	Rank	(OF)	Rank	Combined	Rank	(OS)	Rank	(OF)	Rank	(OS)	Rank	(OF)	Rank	Combined	Rank
Dabo banja	0.62	1	1.08	2	111.35	1	137.41	2	88.39	7	122.8	1	100.28	2	147.51	1
Gibe	0.56	2	1.10	1	88.18	2	140.55	1	112.62	2	110.08	2	101.39	1	116.81	2
Tseday	0.55	3	0.95	4	87.22	3	132.29	3	88.73	5	107.64	3	88.33	4	115.54	3
Etsub	0.42	6	1.04	3	73.95	16	99.14	5	116.31	1	83.2	6	95.83	3	97.96	16
Amarech	0.54	4	0.88	9	86.18	4	129.21	4	77.51	15	106.46	4	81.48	9	114.16	4
Gimbichu	0.37	12	0.95	5	78.55	8	68.19	12	97.34	3	72.79	12	88.29	5	104.05	8
Simada	0.45	5	0.87	12	81.07	5	97.77	6	77.78	14	88.55	5	80.09	12	107.4	5
Keytena	0.41	7	0.88	10	76.25	13	84.87	7	85.28	8	80.53	7	81.02	10	101	13
Ziquala	0.37	11	0.88	8	76.64	12	72.01	11	82.94	9	72.89	11	81.71	8	101.53	12
Welenkomi	0.34	17	0.90	6	74.72	14	59.66	22	93.05	4	67.22	17	83.33	6	98.97	14
Genete	0.34	19	0.87	11	78.31	10	58.43	25	79.93	12	66.79	19	80.56	11	103.74	10
Gerado	0.36	13	0.85	14	78.09	11	63.05	16	80.59	11	71.43	13	78.24	13	103.44	11
Dimma	0.41	8	0.80	17	78.77	7	80.42	8	68.98	20	80.18	8	73.61	17	104.34	7
Mechare	0.37	10	0.83	15	78.35	9	66.86	13	76.51	17	73.74	10	76.39	15	103.78	9
Gemechis	0.31	24	0.89	7	71.95	19	55.4	27	88.65	6	61.85	24	81.94	7	95.31	19
Dega Tef	0.32	23	0.85	13	70.29	22	59.73	21	80.6	10	62.35	23	78.24	14	93.13	22
Melko	0.35	15	0.78	18	74.43	15	59.53	23	72.56	19	68.07	15	72.22	18	98.59	15
Magna	0.30	28	0.80	16	66.28	23	57.09	26	77.04	16	59.88	28	73.75	16	87.81	23
Zobel	0.34	16	0.76	20	70.41	21	62.06	19	72.58	18	67.91	16	69.91	20	93.26	21
Dukem	0.30	27	0.78	19	63.09	26	58.92	24	78.71	13	60.02	27	71.76	19	83.57	26
Koye	0.33	21	0.70	21	73.46	17	60.35	20	54.91	23	65.51	21	64.81	21	97.31	17
Asorgi	0.39	9	0.63	25	73.06	18	77.88	9	48.16	26	77.64	9	57.87	25	96.77	18
Enatit	0.36	14	0.66	22	79.98	6	62.65	17	45.21	27	70.53	14	60.65	22	105.95	6
Ambotoke	0.34	18	0.65	23	71.93	20	64.83	15	48.91	25	67.08	18	60.19	23	95.29	20
Holeta Key	0.31	25	0.64	24	56.99	28	65.97	14	61.32	21	61.53	25	59.26	24	75.49	28
Yilmana	0.32	22	0.62	27	61.76	27	62.63	18	53.58	24	63.12	22	56.94	27	81.8	27
Menagesha	0.31	26	0.62	26	65.45	24	50.19	28	58.14	22	61.3	26	57.41	26	86.68	24
Quncho	0.34	20	0.59	28	64.27	25	73.16	10	42.79	28	66.19	20	54.54	28	85.15	25
Mean	**0.3836**		**0.814**		**75.39**		**77.2**		**75.3**		**75.62**		**75.4**		**99.9**	
F-statistic	**16.72**		**3.81**		**6.06**		**20.4**		**6.22**		**16.72**		**3.81**		**6.05**	
P-value	**<.001**		**<.001**		**<.001**		**<001**		**<001**		**<001**		**<001**		**<.001**	
LSD (0.05)	**0.0606**		**0.209**		**11.902**		**17.14**		**21.81**		**11.96**		**19.44**		**15.78**	
CV (%)	**7.7**		**12.6**		**11.1**		**10.8**		**14.1**		**7.7**		**12.6**		**11.1**	

GY-UL-grain yield from unlimed plots; OS-On-Station; OF-On-farm; STI 1-4 stress tolerance indices 1-4 as indicated in section 4.2.2.2.

Table 4.8. Summary of combined ANOVA for soil acidity tolerance indices of above-ground biomass of on-station and on-farm field experiments

Source of variation	Df		UL AGBM	STI-1	STI-2	STI-3	STI-4
Block	1						
Variety	27	F-statistic	12.58	3.92	28.44	12.51	3.84
		P-value	<.001	<.001	<.001	<.001	<.001
Site	1	F-statistic	300.52	34.99	52.14	57.54	0.27
		P-value	<.001	<.001	<.001	<.001	0.604
Variety. Site	27	F-statistic	1.7	1.32	3.37	1.54	1.37
		P-value	0.048	0.191	<.001	0.089	0.16
Residual	55						
Total	111						

UL-Unlimed; AGBM- above-ground biomass yield, STI-1-4- Stress tolerance indices 1-4 , as described in section 4.2.2.2

4.3.3 Correlations between pot and field experiments

Correlations between the results from the pot and field experiments were significant. However, correlation analyses between growth responses and tolerance indices of the pot experiment and field experiments were varied. Relative root dry weight (RRDW), and relative shoot length (RSHL) of seedlings showed strong and significant associations with all tolerance indices (Table 4.11). RSHDW also showed significant associations with all tolerance indices, except for GY-STI-2 of the field experiments. Inconsistent correlations between the rest of the seedling growth parameters and tolerance indices, and with field tolerance indices, reflects the importance of other agro-climatic factors affecting crop growth under field conditions more than the greenhouse experiment. The improved varieties tested in these experiments were not evaluated at the current test location before their release. Hence, their poor performance under field conditions could be due to their poor adaptation to the complex edaphic and climatic factors of the test environments. The consistent superiority of the local check, both in the pot and the field experiments, confirms the advantages of using local genetic resources collected from such ecologies in the breeding of acid tolerant or Al-tolerant varieties that are well adapted to these agro-ecologies.

Table 4.9. Acidity tolerance indices of commercial tef varieties computed from their above-ground biomass

Variety	AGBM Unlimed (T.ha^{-1})				STI-1		STI-2				STI-3		STI-4	
	OS	Rank	OF	Rank	Combined	Rank	OS	Rank	OF	Rank	Combined	Rank	Combined	Rank
Dabo banja	4.17	1	5.20	2	106.27	1	142.99	1	140.78	2	122.76	1	139.80	1
Gibe	3.80	2	5.45	1	88.09	3	141.62	2	188.61	1	120.36	2	116.00	3
Tseday	2.88	3	3.65	6	89.53	2	77.36	7	85.77	12	85.41	3	118.10	2
Amarech	2.75	4	3.39	13	87.34	4	70.94	12	77.14	19	80.35	8	115.40	4
Dimma	2.68	5	3.75	4	86.99	5	69.09	13	93.07	5	83.69	4	114.80	5
Etsub	2.60	6	3.70	5	74.80	15	83.97	5	95.89	3	82.00	6	98.00	15
Simada	2.55	7	3.39	13	83.07	6	67.67	14	77.14	18	77.52	10	109.40	6
Melko	2.53	8	2.75	19	69.55	24	75.37	8	64.38	22	69.57	20	92.00	22
Holeta Key	2.50	9	3.85	3	78.77	9	83.74	6	89.60	7	82.34	5	102.30	10
Gerado	2.48	10	3.25	14	79.79	8	63.31	18	77.64	17	74.81	14	105.40	7
Keytena	2.48	11	3.75	4	70.62	22	90.55	3	95.55	4	80.78	7	91.80	23
Gemechis	2.45	12	3.55	8	74.97	14	74.06	9	90.05	6	78.03	9	98.40	14
Ziquala	2.40	13	3.40	12	79.96	7	65.95	15	76.78	20	75.51	12	104.80	8
Koye	2.35	14	2.35	20	63.09	27	72.86	10	51.72	28	62.25	26	83.40	27
Gimbichu	2.33	15	3.55	8	74.17	16	71.61	11	85.95	11	76.21	11	96.80	16
Quncho	2.33	16	2.88	17	60.02	28	87.65	4	69.57	21	68.15	22	78.30	28
Mechare	2.28	17	2.95	16	75.63	13	64.35	17	59.71	24	68.32	21	99.00	13
Welenkomi	2.20	18	3.60	7	76.71	12	61.39	19	86.26	10	74.99	13	100.20	12
Dega Tef	2.14	19	3.53	9	73.15	18	55.34	21	84.49	14	73.15	15	96.10	18
Enatit	2.11	20	2.95	16	78.65	10	54.24	22	56.38	27	65.85	24	102.70	9
Genete	2.03	21	3.43	11	76.91	11	49.43	27	80.08	15	70.35	17	100.70	11
Yilmana	2.03	22	2.80	18	64.15	25	64.73	16	59.88	23	62.89	25	83.50	26
Asorgi	1.98	23	2.80	18	70.82	21	50.46	26	58.71	25	62.17	27	92.80	21
Dukem	1.98	24	3.53	9	73.04	19	51.95	24	86.37	9	70.82	16	95.40	20
Menagesha	1.98	25	3.15	15	64.01	26	51.43	25	88.74	8	66.34	23	84.40	25
Ambotoke	1.95	26	2.80	18	72.93	20	46.61	28	58.23	26	61.80	28	95.80	19
Zobel	1.95	27	3.48	10	74.14	17	53.80	23	77.88	16	69.86	19	96.30	17
Magna	1.90	28	3.55	8	69.73	23	56.08	20	84.78	13	70.02	18	90.40	24
Grand mean	**2.419**		**3.44**		**76.32**		**71.4**		**83.6**		**76.3**		**100.1**	
F-statistic	**6.43**		**7.63**		**3.92**		**12.19**		**19.9**		**12.51**		**3.84**	
P-value	**<.001**		**<.001**		**<001**		**<001**		**<001**		**<001**		**<001**	
LSD (0.05)	**0.5954**		**0.686**		**13.679**		**19.44**		**17.59**		**11.619**		**18.45**	
CV	**12**		**9.7**		**12.6**		**13.3**		**10.3**		**10.7**		**13**	

AGBM above-ground biomass from unlimed plots; OS-On-Station; OF-On-farm; STI 1-4 represent stress tolerance indices 1-4 as described in section 4.2.2.2.

Table 4.10. Correlation between tolerance indices and actual yields from limed and unlimed plots (n= 112)

GY- L	1	-										
GY-STI-1	2	0.01NS	-									
GY-STI-2	3	0.21*	0.59***	-								
GY-STI-3	4	0.18NS	0.80***	0.95***	-							
GY-STI-4	5	0.02NS	1.00***	0.59***	0.80***	-						
GY-UL)	6	0.91***	0.41***	0.41***	0.48***	0.42***	-					
AGBM-L	7	0.68***	0.08NS	0.28**	0.25**	0.09NS	0.66***					
AGBM-STI-1	8	0.42***	0.50***	0.43***	0.51***	0.51***	0.55***	0.18NS	-			
AGBM-STI-2	9	0.35***	0.42***	0.58***	0.59***	0.42***	0.49***	0.78***	0.53***	-		
AGBM-STI-3	10	0.44***	0.51***	0.60***	0.63***	0.51***	0.60***	0.64***	0.80***	0.93***	-	
AGBM-STI-4	11	0.02NS	0.54***	0.49***	0.56***	0.54***	0.20*	-0.09NS	0.90***	0.45***	0.70***	-
AGBM-UL	12	0.70***	0.41***	0.46***	0.50***	0.42***	0.80***	0.74***	0.78***	0.85***	0.94***	0.54***
		1	2	3	4	5	6	7	8	9	10	11

***= P<0.001; **= P<0.01; *= p<0.05; NS= statistically not significantly different at P=0.05; GY L/UL= grain yield under limed or UL-unlimed, respectively; AGBM-L/UL= Above-ground biomass yield under limed or unlimed plots, respectively; STI-1-4=Stress tolerance index 1-4.

Table 4.11. Correlations between tolerances indices generated in pot and field experiments (n=28)

AGBM-STI-1	1	-														
AGBM-STI-2	2	0.59***	-													
AGBM-STI-3	3	0.79***	0.96***	-												
AGBM-UL	4	0.79***	0.74***	0.84***	-											
ARL-UL	5	0.22NS	0.04NS	0.09NS	0.27NS	-										
ASHL-UL	6	0.45*	0.26NS	0.34NS	0.41*	0.81***	-									
GY-STI-1	7	0.83***	0.56**	0.71***	0.84***	0.49**	0.65***	-								
GY-STI-2	8	0.73***	0.77***	0.84***	0.96***	0.15NS	0.34NS	0.71***	-							
GY-STI-3	9	0.82***	0.75***	0.85***	0.99***	0.28NS	0.48*	0.87***	0.96***	-						
GY-UL	10	0.8***	0.74***	0.84***	1.00***	0.27NS	0.41*	0.83***	0.96***	0.99***	-					
RDW-UL	11	0.31NS	0.06NS	0.13NS	0.305NS	0.90***	0.81***	0.56**	0.17NS	0.33NS	0.31NS	-				
RRDW	12	0.65***	0.40*	0.52**	0.59***	0.63***	0.77***	0.81***	0.45*	0.62***	0.59***	0.77***	-			
RRL	13	0.38*	0.16NS	0.24NS	0.36NS	0.84***	0.82***	0.61***	0.21NS	0.38*	0.36NS	0.77***	0.78***	-		
RSHDW	14	0.61***	0.45*	0.56**	0.49**	0.58**	0.75***	0.70***	0.37NS	0.53**	0.49**	0.64***	0.85***	0.72***	-	
RSHL	15	0.59**	0.46*	0.55**	0.53**	0.63***	0.77***	0.72***	0.40*	0.56**	0.53**	0.67***	0.88***	0.78***	0.91***	-
SHDW-UL	16	0.46*	0.27NS	0.36NS	0.40*	0.74***	0.88***	0.61***	0.33NS	0.46*	0.40*	0.77***	0.76***	0.73***	0.84***	0.72***
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

***= P<0.001; **= P<0.01; *= p<0.05; NS= statistically not significantly different at P=0.05; n= 28; GY L/UL= grain yield under limed or UL-unlimed, respectively; AGBM-L/UL= Above-ground biomass yield under limed or unlimed plots, respectively; STI-1-4=Stress tolerance index 1-4; RRDW=relative root dry weight; RRL=relative root length; RSHW=relative shoot dry weight ; RSHL-relative shoot length; ARL=mean root length; SHDW=shoot dry weight; ASHL=mean shoot length.

4.3.4 Farmers' assessments

Tef is the primary crop grown in the region, followed by potato and barley. Two systems of tef production prevail in the area, based on the growing period of the landraces. In the long season system, the brown seeded, relatively late maturing *Dabo banja,* is widely planted. The planting takes place during the second week of July and the harvesting after the second week of December. The short season system is a component of a double cropping system where the early maturing landrace *Fesso* is planted in the second week of August after harvesting potatoes. This system avoids or escapes frost damage that can occur with the long season system.

Tef is liked for its adaptability to the local soil and the climate, its use as a nutritious grain crop for human food and for the provision of a high quality straw which has many uses including as an excellent animal feed. Overall, it has better composite market value than any competing crop such as potato, barley or triticale.. It is also resistant to field and storage pests, and is less susceptible to hail and mild frost damage than the other crops grown in the area.

The farmers' preferred attributes for tef varieties were: Adaptability to acid soils; high grain yield; high straw yield; tolerance to mild frosts in the late maturing varieties; earliness and good yields for the short season varieties; tolerance to late rain and hail damage; straw palatability and preference by livestock; good milling qualities; and most importantly, for good cooking qualities for *injera,* a trait that is locally and collectively called *bereket*.

The farmers' selection criteria for tolerance to soil acidity were crop establishment or stand (locally called *biqilet*), tillering capacity (locally called *ribbi*) and plant vigour expressed as height. Very early varieties selected to fit into a double cropping system, and relatively early varieties to avoid late season frosts, had additional selection criteria. Good panicle length and panicle branching locally termed as *zala* was also a selection criterion to indirectly select for better grain yields. Based on these criteria, the farmers selected the variety *Gibe* for the long season system and the varieties *Tseday, Simada* and *Amarach* for the short season system, primarily based on their crop establishment and tillering capacity. The variety *Gibe* was late compared to the farmers' benchmark local landrace, *Dabo banja*, but the farmers proposed to plant *Gibe* during the first week of July so that it would mature early, allowing it to escape

late season frosts. The farmers mentioned that *Dabo banja* was inferior to most of the varieties in panicle length and panicle branches.

Correlations between various yield components with grain and above-ground biomass yield under unlimed condition are presented in Table 4.12. Except for days-to-maturity, all the variables showed a strong association with grain yield and above-ground biomass. The number of fertile tillers, plant height, and particular panicle features noted by the farmers may help to select varieties with better adaptability for desirable agronomic traits. The varieties tested in this experiment were not originally selected for the highly acid test environment. Early materials like *Tsedey* gave more grain and above-ground biomass than late maturing varieties. Consequently, days-to-maturity correlated poorly with grain yield and above-ground biomass.

Table 4.12. Correlation between yield and other yield components of the on-farm experiment under unlimed condition

DM-UL	1	-							
GY-UL	2	-0.06 NS	-						
NPB-UL	3	0.27 **	0.68***	-					
PH-UL	4	0.27**	0.78***	0.86***	-				
PNL-UL	5	0.43***	0.59***	0.81***	0.88***	-			
AGBM-UL	6	0.16NS	0.80***	0.66***	0.79***	0.59***	-		
TN-UL	8	-0.20*	0.57***	0.22*	0.39***	0.20*	0.57***	0.51***	-
		1	2	3	4	5	6	7	8

***= P<0.001; **= P<0.01; *= p<0.05; NS= statistically not significantly different at P=0.05; n= 112; UL-Un limed; DM= days to maturity; GY= grain yield; NPB= number of panicle branches per primary panicle; PH=plant height; PNL=panicle length; AGBM= Above-ground biomass; St at Harv= stand at harvest; TN=Number of fertile tillers number.

4.4 Discussion

The results of the pot experiment showed contrasting responses of *E. tef* genotypes to soil acidity when assessed with root and shoot growth parameters and relative tolerance indices. Growth parameters and relative tolerance indices have been used in screening of various crop and forage species for their tolerance of soil acidity (Little, 1989; Foy and Murray, 1998; Hede *et al.*, 2001; Liu, 2005; Dai *et al.*, 2011). In the pot experiment, despite significance correlation between the actual and relative measurements, considerable rank change was observed among the varieties in their tolerance to soil acidity. For instance, *E. curvula* is highly tolerant of Al-toxicity and soil

acidity (Foy *et al.*, 1987; Miles and de Villiers, 1989; Abate *et al.*, 2013). Whilst it ranked 2nd for its tolerance indices, it ranked 27th for growth parameters. Genetically tolerant but slow growing or less vigorous species or varieties tend to have small values for root or shoot growth parameters (Hede *et al.*, 2002). A similar pattern was observed with the local check, *Dabo banja*, that consistently ranked 1st for the relative tolerance index.

The local check *Dabo banja* is a landrace that ranked 1st for all the relative tolerance indices with values of over 100% for all indices. In terms of growth measurements, it ranked 2nd for RDW, 5th for RL and 1st for SHL and SHDW. These ranks showed that the local check is highly tolerant of soil acidity. Landraces from acid soil regions have been the main source of acid tolerant varieties of many crops due to natural and artificial selection associated with soil acidity (Rao *et al.*, 1993; Hede *et al.*, 2001; Stodart *et al.*, 2007; Caniato *et al.*, 2011).

Key Murrie, DZ-01-2785 and *E. pilosa* (Acc 30-5) showed poor tolerance to soil acidity when measured with both growth parameters and relative tolerance indices, with DZ-01-2785 being the most tolerant. These parents were used to develop two sets of recombinant inbred lines [*E. tef* (*Key Murrie*) X *E. pilosa* (30–5)] and [*E. tef* (DZ-01-2785) and *E. pilosa* (30–5)], which were to be used for molecular mapping studies of tef (Solomon, 2007; Assefa *et al.*, 2010). Molecular mapping studies need a mapping population developed from parents with contrasting phenotypes for the trait of interest (Xu and Crouch, 2008; Varshney *et al.*, 2009; Kassa *et al.*, 2010). A lack of contrast for tolerance to soil acidity among these parents ruled out the possibility of using the recombinant inbred-lines developed from these parents for molecular mapping studies of tolerance to soil acidity and Al-toxicity.

Field evaluation is essential in order to test the adaptabilities of test genotypes to complex biotic and abiotic factors other than soil acidity or Al-toxicity (Rao *et al.*, 1993; Hede *et al.*, 2001). The soil acidity tolerance index, expressed as the ratio of yield under unlimed to limed condition, is a commonly used index to determine tolerance to soil acidity of genotypes under field conditions (Carver and Ownby, 1995; Johnson *et al.*, 1997). On the other hand, several indices have been used to identify genotypes tolerant to drought in a range of crop species (Shirani and Abbasian, 2011; Khalili *et al.*, 2012; Abdi *et al.*, 2013; Abdolshahi *et al.*, 2013). Based on their performance in stressed and non-stressed environments, the tested genotypes could be grouped in

to four groups: (a) genotypes that showed superiority under both stressed and non-stressed conditions; (b) genotypes that performed better only under non-stressed conditions; (c) genotypes that performed better only under stressed conditions; and (d) genotypes that performed poorly under both stressed and non-stressed conditions. Measurement of growth parameters under un-limed conditions helped to identify Group c genotypes. A limitation with the separate use of relative acidity tolerance indices is the fact that they may misidentifies Group d genotypes (Rao *et al.*, 1993; Hede *et al.*, 2001).

In this study, the parameters used were: Growth under unlimed condition; relative acidity tolerance, STI-1, and STI-2 of Fernandez (1992) and its modification STI-3, and STI-4, derived from Fischer *et al.* (1998). Due to a nearly perfect association between STI-1 and STI-4, STI-4 was removed from use. The correlation analysis between stress tolerance indices and growth parameters under unlimed conditions showed strong associations. On the other hand, most of the indices did not show significant associations with growth measurements under limed conditions. All the tolerance indices identified the same varieties, with slight changes in ranks. STI-2, however, identified some varieties that were ranked low by the other tolerance indices. STI-2 is peculiar in that it selects genotypes that perform well under both non-stressed and stressed conditions (Fernandez, 1992).Tolerance indices of above-ground biomass and grain yield also showed significant associations under field conditions. Such associations have also been reported in similar studies (Valle *et al.*, 2009).

Correlations between relative tolerance indices of the pot experiment and most of the tolerance indices of the field experiment were significant. Such associations have helped to develop acid tolerant lines used in several breeding programmes (Rao *et al.*, 1993; Gallardo *et al.*, 1999; Hede *et al.*, 2001). Despite the significant correlations, however, there were changes in ranks of the acidity tolerance of genotypes between the pot and the field experiments. These differences could be associated with interaction with other factors that affected the performance of the varieties. For instance, the two varieties, *Mechare* and *Genete*, were ranked 4th and 5th with RRDW of the pot experiment. They were released for drought prone areas of northeast Ethiopia with an altitude of 1450-1850m (MoARD, 2005; MoARD, 2007). These varieties ranked below 10th when they were tested in acid soils at a location which has a high rainfall and an altitude of about 2500m, and cool temperatures (IFPRI and CSA,

2006). None of these varieties were bred for such acid soil ecologies, so the performance of these varieties was inferior to the current mean national yield of 1.5 $t.ha^{-1}$ (CSA, 2015). Since the productivity of the local check was also far below the national mean productivity, its superiority over the improved varieties can be associated with its adaption to the edaphic and climatic conditions of the test location. Despite of the availability of many improved varieties, the local landrace, *Dabo banja,* was being cultivated over a wide area of land in the study area. This confirms the need to develop agronomically superior varieties that are adapted to specific acid soil ecologies.

4.5 Conclusion

In the present study found that there was significant variation for tolerance to soil acidity among the genotypes tested, in both the pot and field trials. Despite significant correlations among tolerance indices, variation in the ranking of germplasm was observed when using the tolerance indices. Growth under limed condition and relative tolerance indices can be used along with other stress tolerance indices that can be selected for their inherent benefits. The results revealed the importance of edaphic and climatic factors, other than soil acidity and Al-toxicity, in justifying the need for field testing of selected genotypes to ensure their adaptability to the target environment. Consistent superiority of the local check over the Released Varieties was observed. However, the grain yield of the 'improved' varieties, as well as the local check, under unlimed condition was far below the national mean for tef yields. This confirms the need to develop acid soil tolerant and agronomically superior varieties adapted to specific acid soil ecologies. Subsequent genetic studies need to develop a new mapping population since there was a lack of adequate contrast for tolerance of acid soils between the three parents of the mapping population used in this study. Farmers selected one late maturing variety (*Gibe*) and three early maturing varieties (*Tsedey, Simada* and *Amarach*) for cultivation on their moderately acidic soils.

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CHAPTER 5

Evaluating the genetic diversity of tef [*Eragrostis tef* (Zucc.) Trotter] accessions collected from sites in Ethiopia with acid soils, using simple sequence repeats (SSR) markers

Abstract

Soil acidity is one of the major crop production constraints in Ethiopia. Tef is among the most widely grown crops in areas of the country with acid soils. The adoption of released tef varieties in areas with acid soils, combined with lime applications, and the rapid expansion of acid tolerant crops such as oat and triticale, threaten tef genetic diversity in these areas. The aim of this study was to assess the extent of genetic diversity among and within tef populations collected from areas of Ethiopia affected by acid soils, using selected and highly polymorphic SSR markers. The SSR markers used were effective at discriminating between the tef genotypes examined. Analysis of molecular variance (AMOVA) showed highly significant differences ($P<0.001$) among and within populations. Among populations and within population variance contributed 9% and 60% of the total genetic variance, respectively. Despite the wide geographical separation of the collection sites, 88.5% of the acid soil accessions were grouped into two clusters (Clusters II and III), while 90% of the germplasm designated as Breeding Materials and Released Varieties were grouped into Cluster-I. A significant degree of genetic differentiation was observed among the populations. The Accessions from the north western Ethiopia exhibited significant level of variation for most of the genetic diversity parameters evaluated. The number of private alleles was significantly higher for the accessions from collected from acid soils than the Released Varieties or the Breeding Materials. Pairwise estimates of genetic identity and gene flow showed high values between Released Varieties and Breeding Materials. The implications of this study on breeding for tolerance to soil acidity, and conservation of genetic resources from areas with acid soils are discussed.

Key words: genetic diversity, soil acidity, SSR markers, tef

5.1 Introduction

Tef [*Eragrostis tef* (Zucc.) Trotter] is an allotetraploid (2*n* = 4*x*= 40), herbaceous, annual crop grown as a staple food crop in Ethiopia. It belongs to the family Poaceae, subfamily Eragrostoideae, tribe Eragrosteae and genus *Eragrostis*. This genus contains about 300 species (Costanza, 1974), but *E. tef* is the only species currently grown as a food crop for its grain. Wide genetic diversity within the genus *Eragrostis* exists mainly in Ethiopia (Vavilov, 1951; Costanza, 1974; Ketema, 1993). Tef is a versatile crop and can be grown under a wide range of climatic and edaphic conditions (Ketema, 1993; NRC, 1996).

Tef is among the important crops affected by soil acidity in Ethiopia (Mamo and Killham, 1987; Mamo *et al.*, 1996). Oat (*Avena sativa* L.) is now grown in areas of the central highlands of Ethiopia that are affected by acid soils, and areas of oat cultivation have expanded rapidly, replacing the crops that used be grown in these areas (IBC, 2007). Cultivation of another acid tolerant crop, triticale (X *Triticosecale* Wittmark), is also expanding rapidly. Together, they threatening the genetic diversity of traditionally grown crops that used to be grown in high rainfall areas prone to acid soils. In addition, the national extension service is currently promoting lime use combined with the cultivation of white seeded, Released Varieties of tef in these areas of the country. Such developments threaten the existence of brown seeded local landraces in areas with acid soils (Belay *et al.*, 2008) and were grounds for collection and conservation of tef germplasm in Ethiopia (Zeid *et al.*, 2012).

The prevalence of brown seeded tef landraces in areas affected by acid soils and altitudes of over 2400m has been documented (NRC, 1996). Nevertheless, the underlying reason for the dominance of brown seeded landraces in such areas has not been determined previously. Soil acidity and associated nutrient imbalances are known to affect genetic diversity of plant species (Houdijk *et al.*, 1993; Roem and Berendse, 2000). Several studies also suggested that when soil acidity acts as a natural or artificial selection force, it can result in the dominance of acid tolerant species and selections within species (Hede *et al.*, 2001; Stodart *et al.*, 2007; Caniato *et al.*, 2011).

Analysis of the genetic diversity present within germplasm collections and among breeding materials is critical for the effective conservation and exploitation of genetic

resources in plant breeding programmes. In tef, genetic diversity studies have been undertaken using morpho-agronomic characteristics (Assefa *et al.*, 2000; Assefa *et al.*, 2001; Assefa *et al.*, 2002; Assefa *et al.*, 2003a; Adnew *et al.*, 2005) and molecular markers such as amplified fragment length polymorphism (AFLP) (Bai *et al.*, 1999; Ayele and Nguyen, 2000); randomly amplified polymorphic DNA (RAPD) (Bai *et al.*, 2000), inter simple sequence repeats (ISSR) (Assefa *et al.*, 2003b), expressed sequence tags containing simple sequence repeats (EST-SSR), single nucleotide polymorphism (SNP), indel and intron fragment length polymorphisms (IFLP) (Yu *et al.*, 2006), and simple sequence repeats (SSR) (Zeid *et al.*, 2011; Zeid *et al.*, 2012). Compared to other DNA markers, the SSR markers were highly polymorphic and could detect high levels of diversity among tef accessions (Zeid *et al.*, 2011; Zeid *et al.*, 2012). SSRs are widely used markers in genetic diversity studies of major cereals due to their high reliability, co-dominance in inheritance, their high polymorphism, and their abundance and good genome coverage (Varshney *et al.*, 2005; Collard and Mackill, 2008).

There is no information with regards to the extent of genetic variation among and within tef accessions collected from areas in Ethiopia with acid soils using molecular markers. Previous studies were primarily focused on molecular diversity studies of a limited genetic pool that were selected and assembled by Ebba (1975), based on morph-agronomic traits variation. The objective of this study was to assess the extent of genetic diversity among and within dominant phenotypes of tef accessions collected from areas affected by acid soils using selected and highly polymorphic SSR markers.

5.2 Materials and methods

5.2.1 Genetic stock

Tef accessions originally collected from areas affected by acid soils were sourced from the Institute of Biodiversity Conservation (IBC), Addis Ababa, Ethiopia. Accessions that had adequate “passport” data and were from areas with a prevalence of highly acid soil were selected and planted at the Adet Agricultural Research Centre during the 2012 cropping season. Dominant phenotypes (selections with the highest phenotypic frequency) were selected based on panicle colour and panicle form to represent each accession. In addition, ten Districts with widespread and high soil acidity problems were selected from the north-western, western and southern parts of

the country, based on secondary data. Twenty seven dominant phenotypes representing the ten Districts were selected for the present study (Table 5.1). Together with these accessions, sixteen additional genotypes were evaluated. These were 9 designated as Released Varieties (6 grain and 3 pasture); 6 designated as Breeding Materials and a relative (*Eragrostis curvula* (Schrad.) Nees var. Ermelo), totalling 42 genotypes for this study. Detailed information on the varieties is presented in Table 5.1.

5.2.2 DNA extraction

DNA extraction was undertaken according to Mbogori *et al.* (2006) and Adugna *et al.* (2011). Five seedlings per genotype were planted in pots in a greenhouse. Leaves were collected from healthy and vigorous one month old seedlings during the morning hours. Proportionally sampled and bulked leaves of five individual seedlings per genotype were expressed onto labelled FTA cards (www.Sigma-Aldrich.com) wrapped in polythene bags. Pestles were used to press the leaf sample until both sides of the FTA card were soaked with a leaf extract. New polythene sheet coverings were used for each of the samples. The pressing board and pestle were rinsed with 70% ethanol between each sample to avoid cross-contamination. Finally, the FTA cards were hung at room temperature for 4 h then stored in paper bags until amplification.

5.2.3 PCR amplification

The DNA was extracted from bulked samples from the FTA cards per sample at the INCOTEC laboratory in South Africa (www.incotec.com). Sixteen highly polymorphic SSR primer-pairs, including 4 EST-SSR markers from the tef genome (Zeid *et al.*, 2012) were selected and used for amplification. PCR products were fluorescently labelled and separated by capillary electrophoresis on an ABI 3130 automatic sequencer (Applied Biosystems, Johannesburg, South Africa).

5.2.4 Data analysis

5.2.4.1 Genetic diversity analysis

Two approaches were adopted to investigate the genetic structure and diversity among the tef genotypes. In the first approach, polymorphisms were treated as binary data (present or absent). However, to determine the genetic structure within and among accessions, a second approach was adopted, based on the co-dominant

nature of the marker, using GENALEX version 6.5 (Peakall and Smouse, 2012). The X^2 test was performed to determine the differences in allele frequencies among the SSR markers. The total number of alleles per locus (N_a), the number of effective alleles per locus (Ne), the observed heterozygosity (H_o), the mean gene diversity (unbiased) (H_e) and inbreeding coefficient (F) were determined using the protocol of Nei and Li (1979).The percentage of polymorphic loci was estimated for each predetermined group, based on areas of collection. Allelic richness (Ar) was corrected for sample size differences and was estimated by using the rarefaction method implemented in HP-Rare 1.0 (Kalinowski, 2005). Global and pairwise F_{ST} were computed by Weir and Cockerham's Q methods using [Q]FSTAT (Weir and Cockerham, 1984). To examine the degree of population differentiation, other parameters such as gene flow and Nei's unbiased genetic distance and identity were estimated using GENALEX. The partitioning of total genetic variation into within and among the areas of collection was done with a molecular analysis of variance (AMOVA) procedure using GENALEX.

5.2.4.2 Cluster analysis

The binary data were used to obtain a dissimilarity matrix using the Jaccard index. The matrix was used to run a cluster analysis, based on Neighbour-joining employing the software DARwin (Perrier and Jacquemoud-Collet, 2006). A dendrogram was then generated on the dissimilarity matrix. Bootstrap analysis was performed for node construction using 10,000 bootstrap values.

Table 5.1. Descriptions of the tef populations and genotypes used in the study

	Population/accession #	Seed Colour	Region	District
Population-I: North western Ethiopia				
1	Acc# 55027	White	Amhara	Sekela
2	Acc# 55028	Light Brown	Amhara	Sekela
3	Acc# 55029	Light Brown	Amhara	Sekela
4	Acc# 55030	White	Amhara	Dangela
5	Acc# 55334	Light Brown	Amhara	Dangela
6	Acc# 55185	Light Brown	Amhara	Banja
7	Acc# 55186	White	Amhara	Banja
8	Acc# 55049	Light Brown	Amhara	Banja
9	Dabo banja	Brown	Amhara	Banja
10	Acc# 242152	Light Brown	Amhara	Ankasha
11	Acc# 238223	White	Amhara	Guzamn
12	Acc# 238224	White	Amhara	Guzamn
13	Acc# 238225	Brown	Amhara	Guzamn
Population-II: Western Ethiopia				
1	Acc# 55146	Light Brown	Oromiya	Nejo
2	Acc# 55154	Brown	Oromiya	Nejo
3	Acc# 55156	Light Brown	Oromiya	Nejo
4	Acc# 207975-1	White	Ben and Gumuz	Asosa
5	Acc# 207975-2	Brown	Ben and Gumuz	Asosa
Population-III: Southern Ethiopia				
1	Acc# 212919	Brown	SNNP	Limo
2	Acc# 212921	White	SNNP	Limo
3	Acc# 212923	Brown	SNNP	Konteb
4	Acc# 225747	Light Brown	SNNP	Chencha
5	Acc# 225750	Light Brown	SNNP	Chencha
6	Acc# 236093	Light Brown	SNNP	Chencha
7	Acc# 227976	Light Brown	SNNP	Sodo Zuria
8	Acc# 237734	Light Brown	SNNP	Sodo Zuria
9	Acc# 212924	Light Brown	SNNP	Sodo Zuria
Population-IV: Released Varieties				
1	*Holeta Key* (DZ-01-2053)	Brown	Grain, Ethiopia	
2	*Gemechis* (DZ-Cr-387/RIL-127)	White	Grain, Ethiopia	
3	*Quncho* (DZ-Cr-387/RIL-355)	White	Grain, Ethiopia	
4	*Tseday* (DZ-Cr-37)	White	Grain, Ethiopia	
5	*Keytena* (DZ-01-1681)	Brown	Grain, Ethiopia	
6	*Mechare* Acc.205953)	White	Grain, Ethiopia	
7	*Witkop* (Pasture-SA)	White	Pasture, S. Africa	
8	*Emmerson* (Pasture-SA)	Brown	Pasture, S. Africa	
9	*SA Brown* (Pasture)	Brown	Pasture, S. Africa	
Population-IV: Breeding Materials				
1	MPSEL-22	White	Mutant line	
2	MPSEL-6	White	Mutant line	
3	*Key Muri*	White	PMP	
4	DZ-01-2785	White	PMP	
5	*E. pilosa (Acc. 30-5)*	Brown	PMP	
6	*E. curvula (var. Ermelo)*	White	Wild relative	

PMP- parents of mapping population; SNNP-Southern Nations and Nationalities and People Region

Table 5.2. Characteristics of 16 SSR markers used in a genetic diversity study of *Eragrostis tef* (Zeid et al, 2012)

No	Marker name	Forward primer	Reverse primer	Repeat type and size	Exp. size (bp)	PIC	Gene diversity
1	CNLTs 11	GTTCATGTGCCTGCGCGTGT	TCCACGGGGAGAGCGACAGA	CT24	216	0.88	0.89
2	CNLTs 33	TTTGCACCTAGTCTCCCATTG	ACGATCGGATGTTTTGCTTT	GA16	230	0.88	0.75
3	CNLTs 42	ATGCATGGATGGATGGCTA	TTACCCAATTGCCCTAGCTG	TC27	179	0.9	0.91
4	CNLTs 60	AGGGTGATAGCTGCCCAGAC	CCCGAGTAATTGGTCGCTAA	TC20&CA7	297	0.86	0.87
5	CNLTs 133	GGGGAGACTGCATTGGACTA	CAAGAGGGACTGCACAGTGA	GA9	249	0.88	0.89
6	*CNLTs 136	TGAGAAGGTAATAACTGGTGAAGC	CAAGGTTTACACACCGTGACTT	CT18	246	0.79/0.88	0.81/0.89
7	CNLTs 157	GGATCCGACATGACGTGTAGT	CACAGAATGAGATTGGGGAGA	CT18	168	0.77	0.79
8	CNLTs 216	GGAAATTCGCACGAGAGAGA	CGAGAGAGAAGCCTGTGAGG	GA15AAG6	191	0.78	0.8
9	CNLTs 255	TCTCAGCATCGTCTTTGTGTG	TTTTGTGCACGTATTTTTGGA	GA15	187	0.86	0.87
10	CNLTs 295	CTCTAAACCCATGACCCCTTC	GGGGAACATAGTTTGAACTTTTA	GT22	182	0.79	0.81
11	CNLTs 380	ACTGCAACGACAACGCTATG	GGGTACATTCGCGAAAAGAG	CT19	223	0.82	0.84
12	CNLTs 416	AACAGATACAGTTGGAGACAGAAATG	CTCTGAGTGCGTCGCAAG	AG19	151	0.75/0.82	0.78/0.84
13	CNLTs 438	CTAACCGGCGGCGAGAGA	CTGCCACATGCGTCGTTAGA	GA14	153	0.78	0.81
14	CNLTs 455	ACTCCGGAAGAACCACAACA	ACATGGAAAGAGGTGGCAAG	GA10 GG GA5	220	0.82/0.82	0.84/0.84
15	CNLTs 484	GAGATCCTACCACGGCGATA	CGCTTTCCCCTCCTTTTGTA	GA18	157	0.68/0.87	0.73/0.88
16	CNLTs 538	CCATCTTAGCTTTGGCGAGA	ACAAGAGGCAACAAGCCAGA	AG18AGA20	176	0.87	0.88
PIC= polymorphic information content; Exp.= Expected size of DNA fragment in base pairs							

5.3 Results

5.3.1 Genetic diversity within and among tef genotypes

All the 16 primer-pairs successfully amplified DNA fragments of the expected size (Tables 5.2 and 5.3). This indicates that the use of FTA cards and the procedures followed were successful for the extraction and preservation of DNA from tef. Higher frequencies of null alleles (non-amplified) were observed for CNLTs11, CNLTs33, and CNLTs455. Among the genotypes assessed in the panel, the highest frequency of null alleles was observed for *E. curvula*. In this study, only five SSR markers, CNLTs11 CNLTs11 CNLTs60, CNLTs255, CNLTs416 and CNLTs438, produced amplicons of the expected size for this species. Consequently, *E. curvula* was removed from the subsequent diversity analysis.

The SSR markers generated a total of 148 alleles (different fragment sizes) and this allowed for the estimation of genetic diversity among the 41 genotypes (Table 5. 3). The number of alleles revealed by each marker ranged from 2 (CNLTs60) to 27 (CNLTs42), with a mean of 9.25. The genetic variability, measured by gene diversity (H_e) varied between 0.02 (CNLTs60) to 0.95 (CNLTs42).These two markers were genic or EST-SSR markers from the coding region of the tef genome. Variation across the 16 SSR loci, estimated by the mean polymorphic information content (PIC) was 0.65, indicating a high level of genetic variation. On the basis of individual loci, the PIC varied between 0.02 (CNLTs60) and 0.95 (CNLTs42). Over 80% of the SSR-loci had PIC values of >0.50 and about 69% of the loci had PIC>0.70, indicating the effective discriminatory power of the individual SSR loci used in the study. Allelic richness was reflected by the number of variant alleles at a locus, which ranged between 1.13 (CNLTs60) and 9.03 (CNLTs42) with a mean value of 4.78. Observed levels of heterozygosity per locus ranged from 0.0 to 0.85, with a mean of 0.23, i.e., only 23% of the loci were heterozygous and the remaining loci reached an acceptable level of homozygosity. The fixation index *F* (also called the inbreeding coefficient) exhibited contrasting values, ranging from 0.01 to 1.0, with an overall mean of 0.55, and 44% of the loci being completely homozygous. The mean expected heterozygosity value of 0.67, suggests that 67% of the genetic individuals could be expected to be heterozygous at a given locus under random mating.

Table 5.3. Genetic diversity parameters of 41 tef genotypes assessed by 16 SSR markers

SSR-Loci	Size range of alleles	Genetic parameters						
		N_a	N_e	A_r	H_o	H_e	F	PIC
[a]CNLTs11	155 - 175	3	2.43	2.89	0.00	0.60	1.00	0.59
[a]CNLTs33	230 - 270	6	3.57	4.30	0.00	0.74	1.00	0.72
[a]CNLTs42	179 - 215	27	19.10	9.03	0.63	0.96	0.33	0.95
[a]CNLTs60	150 - 253	2	1.02	1.13	0.02	0.02	0.01	0.02
CNLTs133	249 - 280	9	1.89	3.56	0.47	0.48	0.01	0.50
CNLTs136	246 - 280	7	5.26	5.76	0.18	0.85	0.78	0.81
CNLTs157	168 - 195	16	10.67	7.60	0.55	0.92	0.39	0.91
CNLTs216	191 -220	7	4.33	4.85	0.00	0.78	1.00	0.77
CNLTs255	85 - 240	8	1.36	2.45	0.27	0.27	0.01	0.27
CNLTs295	182 - 215	7	4.60	4.96	0.00	0.79	1.00	0.78
CNLTs380	223 - 245	10	5.55	5.91	0.00	0.83	1.00	0.82
CNLTs416	151 - 182	6	3.57	4.37	0.00	0.73	1.00	0.72
CNLTs438	85 - 195	6	1.26	2.10	0.22	0.21	0.08	0.20
CNLTs455	220 - 245	5	3.50	4.12	0.00	0.73	1.00	0.71
CNLTs484	157 - 195	16	7.17	6.90	0.85	0.87	0.02	0.86
CNLTs538	176 - 220	13	7.06	6.52	0.49	0.87	0.43	0.86
Mean		9.25	5.15	4.78	0.23	0.67	0.55	0.66
SE		1.55	1.13	0.53	0.07	0.07	0.11	0.07

;[a]- EST-SSR. N_a- total number of alleles per locus; N_e- number of effective alleles per locus; A_r- allelic richness; H_o-observed gene diversity within genotypes; H_e- unbiased expected heterozygosity; F- fixation index; PIC- polymorphic information content,

Inter-population analysis of genetic diversity indicated that all measures of genetic diversity (allelic richness, private allele richness, percent polymorphic loci, observed heterozygosity) differed significantly between populations (Table 5.4). Tef accessions from the north western Ethiopia had a higher number of observed and effective alleles over all the loci. Allelic richness, however, was highest for accessions collected from southern Ethiopia. Private alleles, the number of alleles found in a single sub-population, was higher for accessions from north western Ethiopia and was smallest for the Breeding Materials. The Accessions had higher private allele scores than the Breeding Materials and the Released Varieties. The *H_e* or the probability that any two alleles chosen at random from a population differ at a single locus, was significantly higher for accessions from north western Ethiopia, indicating the presence of greater genetic diversity in this specific population. Out of the 16 SSR markers used, 13 or 81% were polymorphic, with PIC of of over 50%. The percentage of polymorphic loci among the populations showed significant variation. The collections from north western Ethiopia were 100% polymorphic across all loci, while accessions collected from the southern sites were less polymorphic (69%). Plants from the north western

area had more alleles, more multilocus genotypes, more polymorphic loci, and higher levels of observed heterozygosity than the other tef populations.

Table 5.4. Summary statistics of genetic parameters calculated from 16 SSR loci in 41 tef genotypes classified by areas of collection

Populations	N	N_a	N_e	A_r	P_{ar}	I	H_o	H_e	P
North western Ethiopia	13	6.00	3.85	2.94	1.07	1.34	0.27	0.66	100.00
Western Ethiopia	5	2.94	2.42	2.38	0.56	0.80	0.18	0.50	75.00
Southern Ethiopia	9	4.00	3.23	3.26	0.59	0.92	0.17	0.47	68.75
Breeding Materials	5	3.06	2.45	2.4	0.37	0.83	0.24	0.51	81.25
Released Varieties	9	4.00	3.15	2.48	0.45	0.97	0.21	0.52	81.25
Mean		4.00	3.02	2.69	0.61	0.97	0.21	0.53	81.25
SE		0.34	0.27	0.17	0.12	0.08	0.03	0.04	5.23

N-Number of individual within each population; N_a-mean number of alleles per locus; N_e-mean number of effective alleles per locus; A_r-allelic richness; P_{ar}-Private allelic richness; I-Shannon's information index; H_o-mean observed heterozygosity with in genotype; gene diversity within genotypes; H_e- mean unbiased expected heterozygosity under Hardy-Weinberg assumptions ; P-percentage of polymorphic loci; SE- Standard error

5.3.2 Distance-based population differentiation

The result of analysis of molecular variance (AMOVA) showed highly significant (P=0.001) genetic variation among tef populations, among genotypes, and within genotypes (Table 5.5). Among population variation explained 9% of the total genetic variance. About 60% of the genetic variation in the present sample could be attributed to variation among genotypes, while 31% was explained by variation within genotypes.

Population differentiation (F_{ST}) measures, among the 41 tef genotypes varied between 0.08 and 0.23 (Table 5.6). According to Wright (1978) the degree of differentiation between Breeding Materials and Released Varieties (0.08); Released Varieties and north western (0.13); and western and north western (0.14) were grouped under moderate level of differentiation. Accessions from western Ethiopia showed a large degree of differentiation from the accessions from southern Ethiopia (0.23), and the Breeding Materials (0.20) and the Released Varieties (0.23). Overall, the degree of differentiation was least between the Released Varieties and the Breeding Materials.

According to Morjan and Rieseberg (2004) and Slatkin (1989), gene flow (N_m) between tef accessions from north western Ethiopia and the rest of the tef populations was high. Gene flow between accessions from southern Ethiopia and the other three populations was low. The highest level of gene-flow (2.72) was observed between the

Released Varieties and the Breeding Materials, which was expected because of the likelihood of physical and genetic mixtures being generated at experimental stations.

Table 5.5. Analysis of molecular variance (AMOVA) among 41 tef genotypes collected from five collection sites, using 16 SSR markers

Source	df	SS	MS	Est. Var.	Per. Var.	F-Statistics
Among Populations	4	63.401	15.850	0.486	9%	0.001
Among genotypes	36	293.087	8.141	3.247	60%	0.001
Within genotype	41	67.500	1.646	1.646	31%	0.001
Total	81	423.988		5.380	100%	

Df- Degree of freedom, SS-sum of squares, MS-mean sum of squares, Est. var.-estimated variance, Per. Var.-Percentage variation

Mean genetic distance, as measured by Nei's unbiased genetic distance (Nei, 1987), was higher between accessions collected from western Ethiopia and the Released Varieties, and was smallest between the Released Varieties and the Breeding Materials. Genetic identity, which is an estimate of the proportion of genes that are identical in two populations, was highest between the Breeding Materials and Released Varieties (0.92), and smallest between the Released Varieties and accessions from western Ethiopia.

Table 5.6. Pair-wise estimates of gene flow (N_m) (above diagonal, within the brackets), genetic differentiation (F_{ST}) (above diagonal without brackets); genetic distance (*GD*) (lower diagonal without brackets) and genetic identity (*GI*) (lower diagonal within the brackets)

	North western accessions	Western accessions	Southern accessions	Breeding Materials	Released Varieties
North western		0.14 (1.55)	0.15 (1.47)	0.16 (1.34)	0.13 (1.68)
Western	0.38 (0.69)		0.23 (0.82)	0.20 (0.99)	0.23 (0.83)
Southern	0.36 (0.70)	0.46 (0.63)		0.19 (1.07)	0.17 (1.20)
Breeding Materials	0.51 (0.60)	0.44 (0.65)	0.34 (0.71)		0.08 (2.72)
Released Varieties	0.40 (0.67)	0.54 (0.59)	0.29 (0.75)	0.09 (0.92)	

N_m= gene flow = 0.25 $(1-F_{ST})/F_{ST}$

Elucidation of relatedness among the tef genotypes by a neighbour-joining algorithm using the unweighted pair group method (UPGMA) revealed three distinct clusters (Figure 5.1). All the Released Varieties, including the three pasture varieties from South Africa and 60% of the Breeding Materials, were grouped under Cluster-I.

Cluster–II consisted 55% of the accessions from southern Ethiopia and 60% of the accessions from western Ethiopia. And 84.6% of the accessions were grouped under Cluster-III. Accessions from the south were more scattered, compared to the other populations. Accessions from western Ethiopia were divided in to two clusters, i.e., Clusters II and III. Cluster II and III consisted 88.5% of the accessions collected from areas with acid soils.

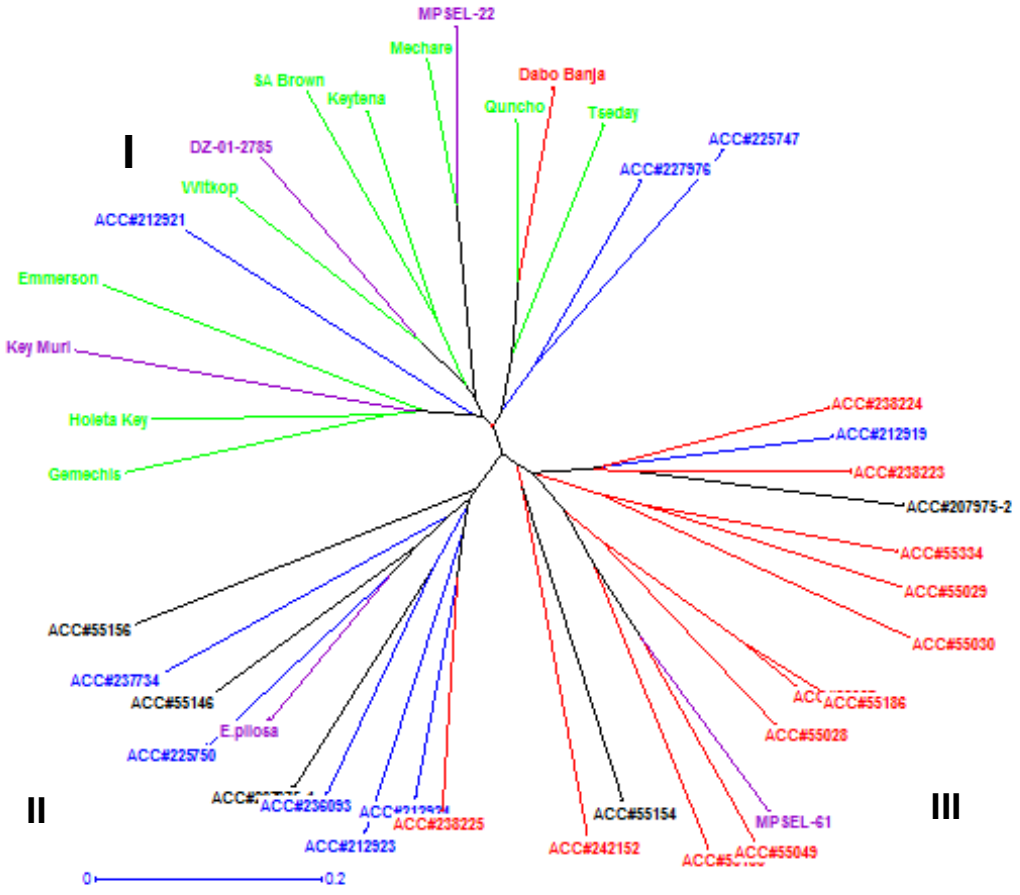

Figure 5.1.Neighbour-joining dendrogram depicting genetic relationship between genotypes and among the five populations. Light green, purple, blue, black and red colours represent Released Varieties, Breeding Materials, and Accessions from the southern, western and north western regions of Ethiopia, respectively.

5.4 Discussion

Successful retrieval and PCR amplification of the DNA fragments in this study verified the value of using FTA cards for DNA extraction, preservation and processing from tef plants. DNA collection on FTA cards does not require special skills and the technology is suitable for field and ecological studies, especially in remote areas and developing

countries. Relative to conventional germplasm exchange methods, FTA-extracted DNA bears little risk of disease transfer, and avoids the danger of unauthorized multiplication and cultivation of precious genetic resources such as that of tef (Mbogori *et al.*, 2006; Adugna *et al.*, 2011).

The high frequency of null alleles observed for *E. curvula* var. Ermelo (from South Africa) is assumed to be associated with species differences. Poor primer annealing due to nucleotide sequence divergence has been suggested as the probable cause of SSR null alleles. Species difference and mutations involving point mutations or indels are implicated in the sequence divergence that results in null alleles (Dakin and Avise, 2004). The other species tested, *E. pilosa,* produced fewer null alleles than *E. curvula*. This was expected because these markers were tested and screened on 151 recombinant inbred lines derived from the cross [*E. tef* (*Kay Murri*) X *E. pilosa* (Acc-35)] (Zeid *et al.*, 2011). Earlier studies showed that *E. tef* had a greater genetic similarity with *E. pilosa* than *E. curvula*. It has been suggested that *E. pilosa* is possibly the closest wild ancestor of *E. tef* (Ayele *et al.*, 1999; Bai *et al.*, 1999; Ayele and Nguyen, 2000; Bai *et al.*, 2000; Ingram and Doyle, 2003). In this study, *E. pilosa* was grouped in Cluster-II with tef accessions, which contrasts with the findings of Zeid *et al.* (2012) that grouped *E. pilosa* accessions along with other wild relatives, using 47 SSR. Like *E. tef, E. pilosa* is an allotetraploid and is the only species that has been successfully crossed with *E. tef*. (Assefa *et al.*, 2010).

Two SSR markers, CNLTs42 and CNLTs60, gave the maximum and minimum values, respectively, for nearly all the genetic diversity parameters, including gene diversity and PIC (Table 5.2). In earlier studies, these markers had gene diversity values of 0.91 and 0.87, and PIC values of 0.9 and 0.89, respectively (Zeid *et al.*, 2012). The low PIC values of CNLTs60, CNLTs255 and CNLTs438 markers in this study suggested that the genetic sequences used for developing these markers were highly conserved in the tef population used in this study.

The high level of discriminating power of the 16 SSR markers used in this study was demonstrated by their ability to differentiate between tef Breeding Materials and Released Varieties related in pedigree. MPSEl-22 and MPSEL-61 were mutant (M_3) lines derived from an ethyl methane sulfonate (EMS) treated variety, *Tsedey* (DZ-Cr-37) (Jöst *et al.*, 2014). These lines were accurately differentiated. (Figure 5.1). Similarly, *Quncho* and *Gamachis,* developed from a cross between *Dukem* (DZ-01-

974) × *Magna* DZ-01-196) (Belay *et al.*, 2008) were also effectively separated. This is consistent with the finding of Zeid *et al.* (2012), who used a larger number of SSR markers to discriminate between tef genotypes related in pedigree.

Contrasting values of fixation index (*F*) was observed among the 41 tef genotypes across 16 SSR loci. Very high *F* values can be expected for tef because the crop is a highly self-pollinated plant with an out-crossing percentage of only 0.2-1.0% (Ketema, 1993). Low *F* values detected in over 25% of the loci can be attributed to allelic multiplicity associated to the allotetraploid nature of the tef genome.

Considerable levels of gene flow were observed between the tef populations. For self-pollinated crops like tef, gene flow is likely to be human mediated (Govindaraju, 2002). The high level of gene flow between the Released Varieties and the Breeding Materials screened in this study was probably associated with human mediated seed movement and the ad hoc creation of mixtures. Zeid *et al.* (2012) also reported mean genetic similarity estimates of 0.79 between two seed lots of 11 tef line fingerprinted for genetic purity. Since seed size of tef is very small (hundred kernel mass = 0.18–0.38 mg vs hundred kernel mass of *Arabidopsis* = 0.17–0.21 mg) (Assefa *et al.*, 2010), preserving complete fidelity of germplasm at experimental stations and seed production schemes is difficult.

For the accessions, the indirect gene flow (N_m) estimates reported here give historical estimates and do not reflect the contemporary variation in gene exchanges between populations (Sork *et al.*, 1999). Hence, the N_m values observed among the different populations could be associated with various factors associated with human movement over a long period of time. N_m inversely relates to F_{ST} (fixation index).. The inverse association between the two parameters was observed in this study. For instance, the smallest F_{ST} was observed between the Breeding Materials and the Released Varieties that had the largest N_m values (Table 5.6).

The result of the inter-population assessment of genetic diversity was that the rural accessions had the widest genetic variation for most of genetic diversity measures. High level of genetic diversity is expected in landraces because they are populations with high levels of genetic variability, having adapted to the natural and anthropological environments of their origin, and are therefore reservoirs of useful traits (Barcaccia, 2010; Xu, 2010).

Among the accessions, tef population from the north western region had the highest values for all the genetic diversity parameters except for private alleles. This was consistent with earlier studies that found the northern part of Ethiopia is an area of high genetic diversity for tef (Bai *et al.*, 1999; Bai *et al.*, 2000; Kefyalew *et al.*, 2000; Assefa *et al.*, 2001; Assefa *et al.*, 2003b).

Analysis of molecular variance (AMOVA) showed that there were highly significant ($P<0.001$) variations between the populations. This was also in agreement with a measurement of a significant degree of differentiation (F_{ST}, 0.08-0.23) and genetic distance (*GD,* 0.09-0.54) between the populations. Cluster analysis also showed a clear pattern of differentiation between the populations, mapping onto the predetermined population structure. The proportion of variance explained by among genotypes variance is usually higher than between populations' variance in self-pollinated crops due to the high level of homozygosity within individual genotypes.

A molecular diversity study conducted on tef populations collected from eight geographical areas using inter simple sequence repeat (ISSR) markers previously showed significant differences between and within populations (Assefa *et al.*, 2003b). A phenotypic diversity study of quantitative traits conducted on different populations of tef belonging to 6 regions and 3 altitudinal ranges also showed highly significant variation between populations of different geographic origins, and between populations from the different altitudinal ranges for most of the quantitative traits studied (Assefa *et al.*, 2001). A similar pattern was also reported from previous study (Tadesse, 1993).

In the present study, the tef populations represented not only different geographical areas but also specific acid soil ecologies that would have contributed to the within population difference observed in earlier morpho-agronomic and molecular diversity studies. Despite wide geographical separation, 88.5% of the acid soil accessions were grouped into two clusters (Clusters II and III) while 90% of the Breeding Materials and Released Varieties were grouped into Cluster-I. Significant degrees of differentiation, and genetic distances were observed between the Accessions (Cluster-II and III), and Breeding Materials and Released Varieties (Cluster-I).

Significantly higher numbers of private alleles were found in the Accessions from acid soil ecologies indicating the presence of distinct genetic features in these accessions.

Soil acidity and associated mineral imbalances are known to select acidophilic or calcifuge species and variants within species (Houdijk *et al.*, 1993; Roem and Berendse, 2000; Pilbeam and Morley, 2007). In crop plants, several studies have documented that most of acid soil tolerant crop varieties have been selected from accessions collected from regions with highly acidic soils of the world (Hede *et al.*, 2001; Stodart *et al.*, 2007; Caniato *et al.*, 2011; Maron *et al.*, 2013; Guimaraes *et al.*, 2014).

On the other hand, the low number of private alleles in the population of Released Varieties can be related to the impact of plant breeding that narrows the genetic base. Selection for economically important traits through plant breeding is known to reduce the frequency of rare alleles and the overall genetic diversity (Fu *et al.*, 2006; Rauf *et al.*, 2010). Driven by the need to enhance crop production, expanded cultivation of Released Varieties has resulted in the replacement of some landraces in Ethiopia (Tsegaye and Berg, 2007). In tef, white seeded and high yielding varieties that rely on optimal external inputs have become a threat to tef genetic diversity (Assefa *et al.*, 2001; Belay *et al.*, 2008; Zeid *et al.*, 2012). The current push by the national extension services for farmers to plant of Released Varieties of tef and other cereals, combined with the application of lime, are direct threats to tef landraces adapted to acid soils. Over 69% of tef accessions that represented the dominant phenotypes collected from areas with acid soils in this study were brown seeded.

5.5 Conclusion

Relatively high levels of genetic diversity were found in the dominant phenotypes of accessions sourced from areas with acid soils, which offer great opportunity for plant breeders to breed for acid tolerant and agronomically superior varieties of tef. This will help to achieve two important goals. Firstly, it will improve food security and the livelihoods of small-scale farmers who do not use lime for the amelioration of soil acidity. Secondly, it will assist in the preservation of rare alleles that may confer acid tolerance under farmers' condition. The lack of adequate "passport data" and a lack of accessions from specific growing environments such as acid soils, are the main shortcomings of the spectrum of tef accessions currently conserved in the national gene bank (Assefa *et al.*, 2001; Assefa *et al.*, 2010; Zeid *et al.*, 2012). Hence, urgent efforts to collect a representative spectrum of accessions from areas with acid soils

are needed to rescue the threatened private alleles present in tef landraces that have evolved to tolerate acid soils. A programme is needed to breed tef for specific adaptation to acid soils. The result of this study identifies the need to include seed from all the regions affected by acid soils, and to maximize the within region sampling while undertaking the collection programme.

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CHAPTER 6

Isolation and characterization of ethyl methane sulphonate (EMS) induced mutants of tef [*Eragrostis tef* (Zucc.) Trotter] for aluminum tolerance and morpho-agronomic traits

Abstract

Soil acidity and Al-toxicity are among the major constraints affecting tef production in Ethiopia. However, research on breeding for tolerance to Al-toxicity in tef is in its infancy in the country. The aim of this study was to isolate and characterize Al-tolerant lines in an EMS-induced M_2 population of tef. An improved tef variety, *Tsedey* (DZ-Cr-37), was previously mutagenized using EMS. About 15000 M_2 seeds were screened under acid soil condition, along with the M_0 seeds of the parent variety *Tsedey* and an Al-tolerant local selection. Strongly acidic soil with an external application of a toxic level of Al-solution was used to maximize the root pruning effect of Al and easily rogue out sensitive phenotypes. Further, seedlings were exposed to moisture stress to maximize selection pressure against sensitive lines. Twenty one M_2 plants with root lengths of greater than the mean plus standard deviation of the tolerant check were selected and planted for seed production. These M_3 plants were characterized for variation for Al-tolerance and morpho-agronomic traits under greenhouse and field conditions, respectively. There were highly significant differences for Al-tolerance between the mutant lines and the parent ($P<0.001$); and between mutant lines and the sensitive check ($P<0.001$). However, non-significant difference was observed between the mutant lines and the tolerant check. Similarly, significant differences were observed between the mutant lines for 16 of the 20 quantitative traits measured. This study is the first to report successful induction of enhanced Al-tolerance in tef by using EMS.

Key words: Al-tolerance; Al-toxicity; ethyl methane sulfonate (EMS); mutation breeding, tef

6.1 Introduction

The global population is projected to reach 9 billion by the year 2050. The world will need 70 to 100% more food to feed this population (FAO, 2009). This in turn requires mean annual increment of 44 million metric tons per year for the coming years. Maximizing productivity of crops through development of high yielding crop varieties is one of the strategic options available to increase crop production and to meet the global food demand (FAO, 2009; Godfray *et al.*, 2010; Tester and Langridge, 2010).

Tef [*Eragrostis tef* (Zucc.) Trotter] (2*n*=4*x*=40) is the most widely produced and consumed cereal crop in Ethiopia. In terms of area of cultivation, it is the leading cereal crop followed by maize and wheat. According to the Central Statistical Authority (CSA) (2015), the area covered by tef during the 20014/2015 cropping season was over 3 million hectares or 30% of the total area occupied by cereals in the country. As a gluten-free cereal, tef is currently gaining popularity worldwide (Spaenij-Dekking *et al.*, 2005). Besides, tef is also grown as a pasture crop in several countries (Assefa *et al.*, 2010).

Aluminium toxicity and other acidity related soil fertility problems are among the major constraints affecting tef production in Ethiopia (Dubale, 2001; IFPRI, 2010).The problem is widespread in the high rainfall areas of the north western, western, southern, and south western parts of the country (Schlede, 1989; Abebe, 2007). Worldwide, development of varieties tolerant of acid soils has been a sound alternative to liming, and other non-genetic management options in the production of globally important crops (Rao *et al.*, 1993; Hede *et al.*, 2001).

Mutation breeding has been used to induce variability and develop improved varieties of various crop species worldwide (Jain, 2005; Mba, 2013). Many officially released mutant varieties have been developed worldwide (Mba, 2013). In tef, mutation breeding was started in 1972 using gamma radiation, with the cooperation of International Atomic Energy Agency (IAEA) and Food and Agricultural Organization of the United Nations (FAO) (Tefera *et al.*, 2001). Due to lack of adequate variation in the natural population, the primary focus of tef mutation breeding has been on the development of lodging resistant phenotypes. Recently, a chemical mutagen, ethyl methane sulfonate (EMS), has been successfully utilized to induce mutation as a component of the reverse genetics approach known as TILLING (Targeting Induced

Local Lesions IN Genome) with the objective developing semi-dwarf tef variants resistant to lodging (Esfeld *et al.*, 2009; Jöst *et al.*, 2014). Several studies reported that EMS produces a large number of (genome-wide) non-lethal point mutations in plants (Till *et al.*, 2003; Greene *et al.*, 2003; Till *et al.*, 2004).

Despite the wide spread problems of soil acidity and Al-toxicity affecting tef, breeding for tolerance to Al-toxicity in tef has not been a research focus in Ethiopia. This research work was conducted in order to screen and characterize Al-tolerant lines in an EMS induced M_3 population of tef.

6.2 Materials and methods

The overall activities conducted in selection and characterization of the mutant lines is presented in figure 6.1.

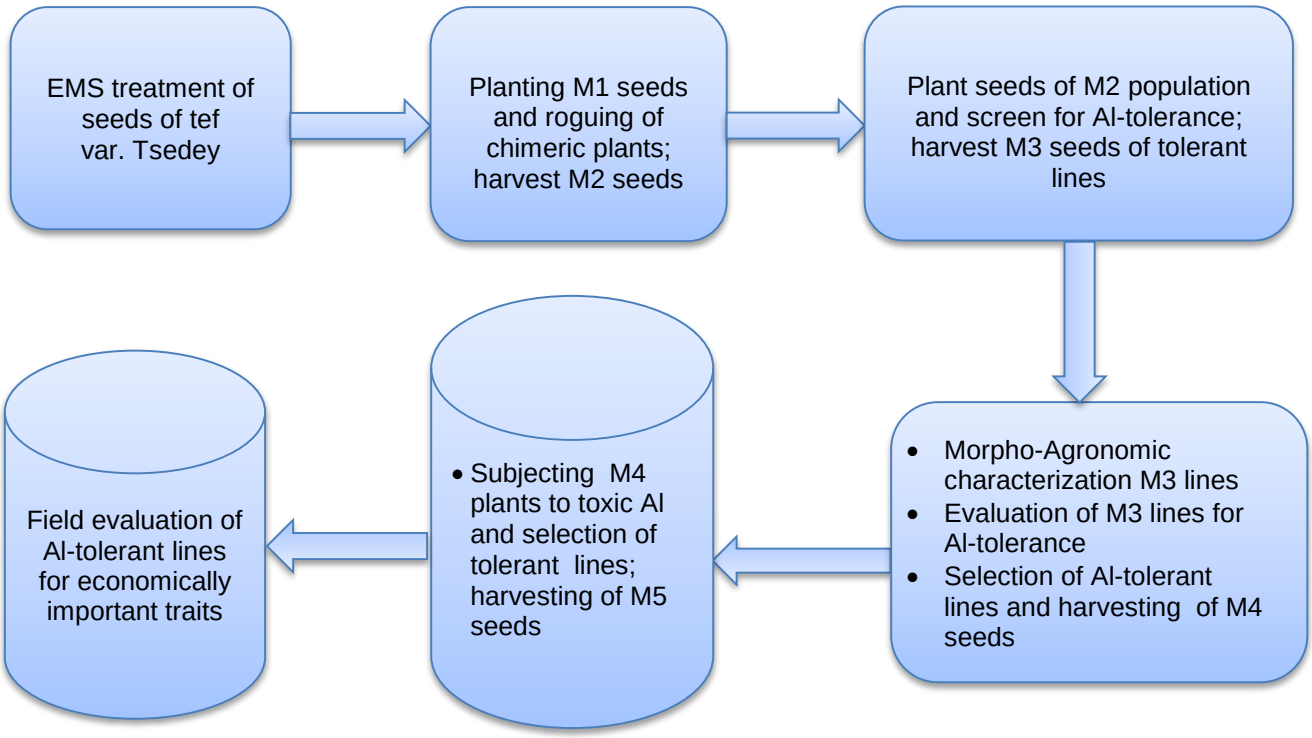

Figure 6.1 Schematic illustration of mutation induction, isolation, evaluation and characterization for Al-tolerance

6.2.1 Induction of mutation

Seeds of an improved tef variety, *Tsedey* (DZ-Cr-37), were mutagenized by the Tef Improvement Project at the Institute of Plant Sciences, University of Bern in

Switzerland, using 0.2% of ethyl methane sulfonate (EMS) for 8 hours. About 10000 plants from the first generation after mutagenesis (M_1 population) were self-pollinated and about 7000 non-chimeric M_2 families were obtained. M_2 seeds pooled from 5000 M_2 families were used for selection on acid soil along with M_0 seeds of untreated parent variety *Tsedey* (DZ-Cr-37) and an Al-tolerant local selection.

6.2.2 Selection of Al-tolerant mutants

Strongly acidic soil with pH (H_2O) 1:2.5 of 4.5 was collected from the major acid soil affected district, Banja. The soil was continuously watered with 222 µM $AlK(SO_4)_2.12H_2O$ until a pH (H_2O)1:2.5 of 4.0 was achieved (Islam *et al.*, 2004). Fifteen thousand M_2 seeds were planted in 30 pots (10cm diameter) in a greenhouse of the Amhara Agricultural Research Institute, at Bahir Dar, Ethiopia. A local landrace with Al-tolerance and the parent were also planted for comparison. The plants were fertilized with NPK at the rate of 100, 109 and 137 µg.g-1 of soil, respectively, using NH_4NO_3 and KH_2PO_4. The pots were uniformly watered with 222 µM $AlK(SO_4)_2.12H_2O$ (pH 4) for the first 2 weeks (14 days). Rogueing of seedlings with poor root development (poorly anchored) was started one week after planting, using fine tipped forceps (Figure 6.2).

The concentration of $AlK(SO_4)2.12H_2O$ was doubled to 444 µm after the second week in order to increase selection pressure. This concentration further differentiated the seedlings and enabled further rogueing during the third week.

Since Al-toxicity impedes root development of sensitive plants, it enhances the vulnerability of such plants to drought of even a short duration. Hence, during the 4^{th} week, green and apparently tolerant seedlings were subjected to moisture stress by discontinuing watering for 96 hours. The Al-tolerant landrace showed wilting after the 4^{th} day. All seedlings of the mutant population and the parent that showed temporary wilting earlier were rogued out. This procedure allowed for the identification of apparently tolerant plants with poorly developed root system. At this stage all the seedlings of the parent variety *Tsedey* were rogued out (Figure 6.3).

Twenty-eight days after planting, the soil was washed and the roots of the Al-tolerant landrace were measured. The mean plus the standard deviation of the root length of the Al-tolerant landrace was used as truncation point to select the Al-tolerant mutant

plants. All mutant plants that had root length of greater the truncation point were transplanted into normal growing medium in pots for seed production (Table 6.1).

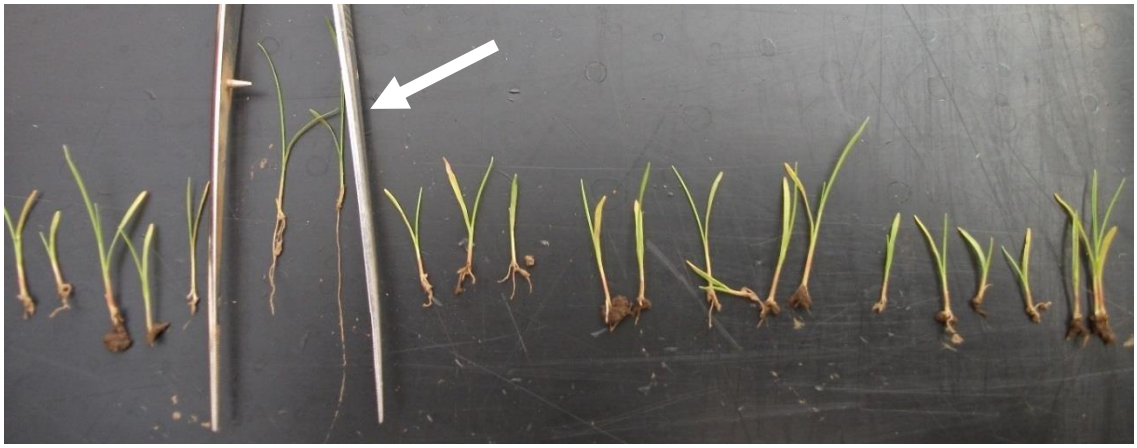

Figure 6.2. Early root pruning effects of Al-toxicity and nutrient deficiency symptoms in sensitive M_3 mutant lines (arrow indicate tolerant selections)

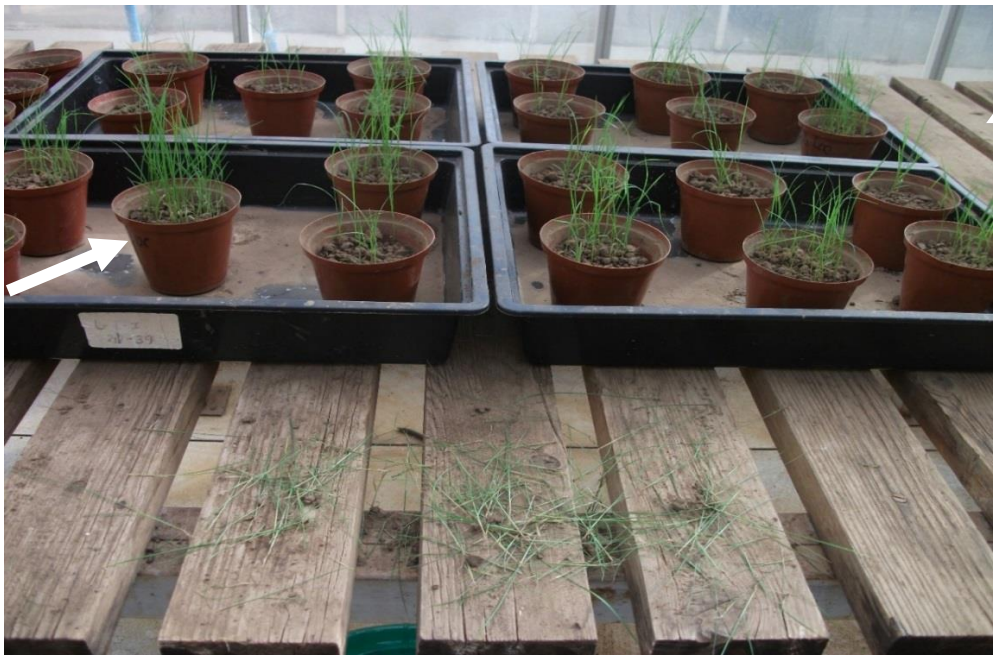

Figure 6.3.Late rogueing of apparently Al-tolerant plants affected by moisture stress due to poor root development; arrows indicate the Al-tolerant landrace.

In order to exclude sensitive segregants, subsequent generations of mutant lines were advanced by subjecting the seedlings to 350µM $AlK(SO_4)_2.12H_2O$. Single plants with the longest root were preserved per mutant line.

Table 6.1. Description of root length of mutant selections compared to the local Al-tolerant selection

Selections	N	Mean	Std.	Min	Max
Al-tolerant landrace	70	34.46	12.35	15	70
Mutant selections measured for root length	217	31.40	11.45	10	70
Mutants selected i.e. above truncation point (47mm)	21	54.60	6.24	47	70

Truncation point= Mean + Std. of Al-tolerant landrace i.e., 47mm; N-number individual plants measured; Std- Standard deviation; Min-Minimum; Max-Maximum.

6.2.3 Evaluation of M_3 lines for Al-tolerance

6.2.3.1 Genetic stock

Twenty one M_3 lines with root length of above 47mm were planted in pots (10cm) in a greenhouse along with M_0 parent *Tsedey* (DZ-Cr-37), an Al-tolerant landrace, and a sensitive check variety, *Holeta Key*.

6.2.3.2 Experimental set up

The experiment was established in randomized complete blocks design with three replications under limed and unlimed condition as described in Sections 4.2.1.2.

6.2.3.3 Data collection and statistical analysis

Data were collected and analysed as described in Sections 4.2.1.3 and 4.2.3.

6.2.4 Morpho-agronomic characterization of mutant lines

6.2.4.1 Experimental set up

The twenty-one M_3 lines, along with M_0 parent *Tsedey* (DZ-Cr-37), were grown at the Adet Agricultural Research centre, Adet, north western Ethiopia, during the 2014 cropping season under natural conditions. A randomized complete block design with two replications was used with a plot size of 0.6 m^2 and inter-row spacing of 20cm. The seeds were drilled in the row with a seed rate of 15 $kg.ha^{-1}$. At tillering, the plants within each row were thinned to an intra-row spacing of 5 cm. Fertilizers were applied with rates of 130 $kg.ha^{-1}$ DAP and 36 $kg.ha^{-1}$ urea. All of the DAP was applied at planting, and all of the urea at tillering.

6.2.4.2 Data collection

Days to 50% panicle emergence; and days to 75% maturity were recorded on a plot basis. Culm length; number of internodes; first basal internode length (cm); first basal internode diameter (mm); second basal internode length (cm); second basal internode diameter (mm); panicle length (cm); number of panicle branches; number of spikelet; number of florets per spikelet; grain yield/panicle (g); and phytomass (gm) were recorded on the basis of the main shoots of seven randomly selected plants from the central row. Counts of spikelet per panicle and the number of florets per spikelet were made for the basal, middle and apical parts of the main shoot panicle.

Number of fertile tillers/plant; grain yield/plant (g), and phytomass yield/plant (g), and the harvest index (%), were recorded on the basis of seven randomly selected plants from the central row. Culm and grains were dried in an oven at 70^{o}C for 48 hours, as described by Hobbs and Sayre (2001), to determine the above-ground biomass and the harvest index. Mean values of these samples were used to describe each line for the traits under consideration.

6.2.4.3 Statistical analysis

Analysis of variance and cluster analysis were performed to assess the variability among the mutant lines and estimate the relatedness among the lines using GenStat Statistical Software Version:17.10013780 (GenStat., 2014).

6.3 Results

6.3.1 Variability for Al-tolerance

Analysis of variance revealed the presence of highly significant differences between the mutant lines for both the tolerance indices and actual measurements under unlimed conditions (Table 6.2). Orthogonal contrast between the parent and the mutant lines also showed highly significant differences for all the parameters. However, the mutant lines and the Al-tolerant landrace, did not show significant differences for all the parameters. Figures 6.4 and 6.5 also showed equivalent shoot and root growth of the tolerant check and the mutant lines. The significant difference observed between the sensitive check and the parent showed that the parent variety was less sensitive to Al-toxicity than the sensitive check.

Table 6.3 shows the responses of the mutant lines in terms of tolerance indices and actual root and shoot growth under unlimed conditions, along with their rank. Relative root dry weight (RRDW) and relative root length (RRL) are better measures of tolerance to Al-toxicity because they indicate the relative performance of the genotype under unlimed conditions and limed conditions. Values of over 100% indicates that the genotype performed well under unlimed conditions relative to limed condition. Compared to RRL, RRDW gives a better measure of tolerance because it takes into account the root density. Accordingly, except for ML99, all the mutant lines were superior to the parent and the sensitive check. This result was expected because the selection was severe and only 0.14% plants from the original 15,000 seeds were retained. The parent variety was significantly less sensitive to Al-toxicity compared to the sensetive check. Similar pattern was observed in previous pot and field experiments (chapter 4) on the performance of these two varieties.

Table 6.2. Analysis of variance for Al-tolerance parameters among mutant lines

Source of variation	d.f.		RRL (%)	RRDW (%)	RL	RDW
Block	2					
TRT	23	P value	<.001	<.001	<.001	<.001
		F-Static	14.26	13.99	4.97	7.07
Parent Vs ML	1	P value	<.001	<.001	0.002	<.001
		F-Static	36.61	27.53	11.19	18.46
Local Vs ML	1	P value	0.059	0.299	0.309	0.34
		F-Static	3.74	1.1	1.06	0.93
Sensitive check Vs ML	1	P value	<.001	<.001	<.001	<.001
		F-Static	102.8	103.5	30.55	79.98
Parent vs Local		P value	<.001	<.001	0.003	0.020
		F-Static	33.39	20.76	10.02	5.81
Parent vs Sensitive check	1	P value	0.005	<.001	0.121	0.002
		F-Static	8.76	12.71	2.49	11.31
Residual	46					
Total	**71**					

ML-mutant lines; RRL-relative root length; RRDW-relative root dry weight; RL-root length unlimed; RDW-root dry weight unlimed.

Figure 6.4. Contrasts between root and shoot growth of the tolerant check, the parent and selected mutant tef lines under unlimed, acid soil conditions

6.3.2. Variability for morpho-agronomic traits

Analysis of variance indicated significant differences for 16 of the 20 morpho-agronomic traits analysed (Table 6.4). The mutant lines did not show significant difference for hundred seed weight, number of internodes, first basal internode diameter and second basal internode diameter. Minimum and maximum values of each traits are presented along with the mean of all the genotypes and the parent variety (Table 6.4).

Agronomically and economically important traits like days to 50% panicle emergence, days to maturity, seed and biomass yield per main shoot and whole plant, panicle length, and number of panicle branches all showed considerable variation around the mean of the parent, suggesting that the mutagenesis and the selection procedures employed have resulted in variability both in positive and negative directions. The maximum whole plant seed yield and whole plant biomass yields of 26.78g and 58.5g were obtained for the selection ML139, a gain of 58.0% and 55.1% over the mean of the parent for both traits, respectively. Similarly, a maximum harvest index of 54.57% was recorded for the selection ML61 with a gain of 22% over the parent.

No difference was observed for most of the qualitative traits between the parent and the mutant lines. But some mutant lines like ML153 were distinct enough in developing an extremely loose panicle form compared to the parent and most of the mutant lines (data not shown). Hierarchical cluster analysis using the Euclidean distance between groups showed that the relatedness among the lines was very close with a maximum dissimilarity value of less than 0.1 for most of the mutant lines (Figure 6.5). The parent

did not show distinct clustering from the mutant lines and was most closely related to the line ML209.

Table 6.3**.** Means and ranks of mutant lines measured in terms of tolerance indices and actual growth under unlimed condition

Mutant lines	RRDW (%)		RRL(%)		RL (mg)		RDW (UL)	
	Mean	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**
ML-209	142.04	1	152.70	2	80.05	3	35.67	1
ML-149	133.85	2	121.10	14	56.9	18	28.50	11
ML-153	119.01	3	137.40	5	84.33	1	32.67	2
ML-96	115.24	4	130.50	10	65	14	31.00	5
ML-207	114.80	5	152.00	3	72.24	8	32.00	3
ML-48	111.52	6	127.20	12	77.95	4	31.83	4
Dabo banja	106.13	7	137.30	6	72.43	7	26.50	16
ML-183	104.59	8	155.60	1	76.71	5	29.33	7
ML-205	102.40	9	136.80	7	68.33	11	29.00	8
ML-184	100.84	10	151.70	4	82.38	2	25.17	18
ML-22	100.14	11	127.30	11	71.67	9	28.33	12
ML-133	98.04	12	133.40	9	68.62	10	28.50	10
ML-117	97.34	13	118.50	15	52.67	20	27.50	15
ML-98	96.94	14	134.90	8	74.14	6	28.33	13
ML-61	93.91	15	110.60	17	66.62	12	29.83	6
ML-94	91.90	16	82.90	21	51.33	21	28.83	9
ML-194	88.74	17	114.30	16	65.33	13	25.00	19
ML-139	87.14	18	84.80	20	54.14	19	24.83	20
ML-148	82.39	19	89.50	18	58.43	16	24.33	21
ML-49	75.25	20	123.30	13	64.05	15	27.83	14
ML-173	68.10	21	85.90	19	57.9	17	26.50	17
T*sedey*	65.41	22	71.00	23	47.58	23	20.17	22
ML-99	61.98	23	76.00	22	49.43	22	20.00	23
Holeta Key	33.55	24	37.00	24	35.19	24	11.33	24
Mean	**95.5**		**116.30**		**64.7**		**27.21**	
LSD (5%)	**17.99**		**23.11**		**15.8**		**5.287**	
CV (%)	**11.5**		**12.10**		**14.8**		**11.8**	

RRDW-relative root dry weight; RRL-relative root length; RL-root length; RDW-root dry weight

Table 6.4. Minimum, maximum and mean values and significance tests of the selected mutant lines of tef for 20 morpho-agronomic traits

No	Trait	Minimum		Maximum		Mean±(SE)	Parent	F-value	P-value
		Value	ML	Value	ML				
1	Days to maturity	98.00	ML207	106.00	ML49 ML99	101.20±(1.68)	99.00	6.45	<.001
2	Days to 50% panicle Emergence	49.00	ML98	57.50	ML49 ML61 ML139 ML153 ML183	54.50±(1.51)	55.00	4.91	<.001
3	Number of fertile tillers	3.67	ML173	10.86	ML139	6.32±(1.22)	5.50	3.69	0.002
4	Main shoot biomass (g)	3.30	ML98	10.86	ML22	6.39±(1.28)	5.65	3.88	0.002
5	Main shoot seed weight (g)	2.50	ML194	6.61	ML61	4.65±(0.89)	5.22	2.51	0.02
6	Whole pant Biomass (g)	24.35	ML48	58.51	ML139	35.78±(3.16)	37.73	13.54	<.001
7	Whole plant seed weight(g)	8.98	ML183	26.78	ML139	14.64±(2.42)	16.93	6.99	<.001
8	Hundred seed weight (mg)	27.00	ML96 ML194	31.00	Parent	28.66± (1.20)	31.00	1.63	0.135
9	Harvest Index	26.55	ML183	54.57	ML61	40.72±(5.60)	44.80	3.05	0.007
10	Plant height (cm)	74.36	ML98	96.14	ML148	84.11±(2.34)	77.32	14.23	<.001
11	Culm length(cm)	43.57	ML207	57.50	ML173	48.69±(2.32)	46.43	3.68	0.002
12	Panicle length	31.43	ML184	42.93	ML61	37.19±(1.99)	34.93	6.94	<.001
13	Number of internodes	2.71	ML117 ML133	3.79	ML139	3.19±(0.54)	3.50	0.61	0.87
14	First basal Internode length (cm)	3.00	ML149	5.06	ML133	3.90±(0.32)	3.46	5.42	<.001
15	First basal Internode diameter (mm)	1.54	ML98	2.15	ML22	1.88±(0.19)	1.96	1.39	0.228
16	Second basal internode length (cm)	6.64	ML96	9.43	ML61	8.04±(0.68)	7.43	2.45	0.023
17	Second basal internode diameter mm)	1.60	ML149	2.11	ML61	1.89±(0.18)	2.02	1.51	0.177
18	Number of panicle branches	21.79	ML98	31.71	ML153	26.08±(2.53)	25.50	2.06	0.05
19	Mean number of florets	5.01	ML61	7.06	ML48	6.03±(0.58)	5.68	2.39	0.026
20	Number of Spikelets per panicle	17.12	ML207	26.81	ML61	21.17±(1.89)	20.60	2.74	0.013

ML-mutant lines; parent is the variety *Tsedey*; F-F statistic or variance ratio; SE-standard error

6.4 Discussion

This study has resulted in the successful isolation of Al-tolerant lines that exceeded the parent in all of the tolerance parameters and actual growth measurements under unlimed conditions. Most of the mutant lines were better or equivalent to the Al-tolerant landrace grown in strongly acidic Acrisols of north western Ethiopia. This suggests that the EMS application has successfully induced variability for Al-tolerance in the original tef population. The screening techniques employed in this study, i.e., combined use of strongly acidic soil along with application of Al in the form of $AlK(SO_4)_2.12H_2O$, and subjecting seedlings to severe drought was efficient at

identifying Al-tolerant lines. This study is the first to report the use of EMS for induction of genetic variability for Al-tolerance in tef. Induction of mutation has been used to increase genetic variability for Al-tolerance in other plants. For instance, Nawrot *et al.* (2001) have reported increased level of Al-tolerance in barley after mutagenic treatment of four varieties with N-methyl-N-nitroso urea (MNH) and sodium azide. Similarly, treatment of Al-sensitive Arabidopsis with EMS resulted in variants that grew in highly toxic Al condition (Kelly *et al.*, 2006).

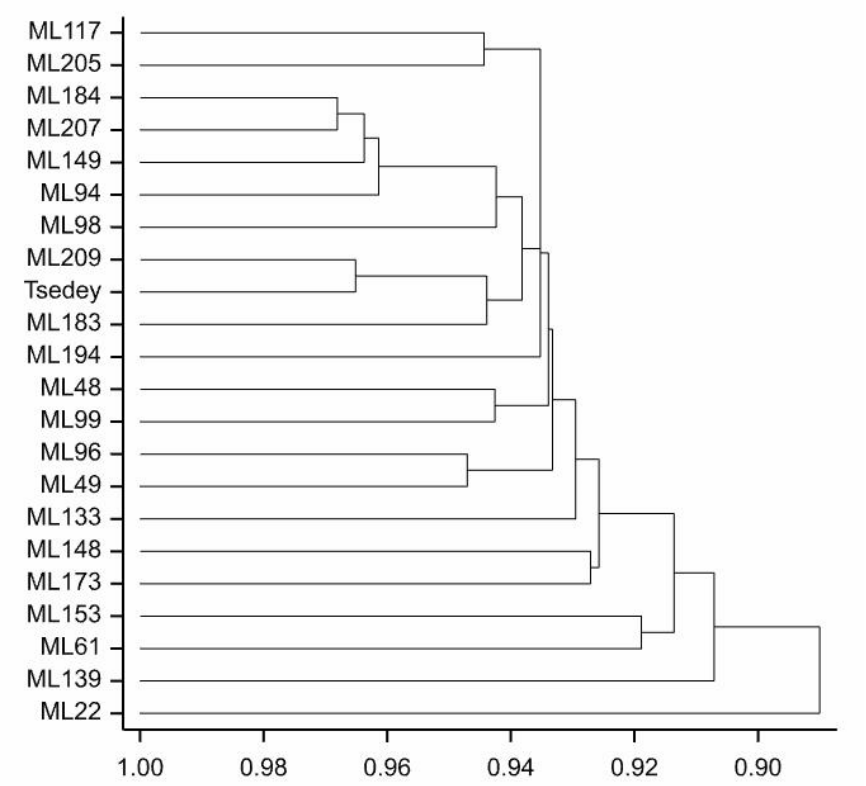

Figure 6.5. Dendrogram showing similiarity among the mutant lines based on 20 morpho-agronomic traits.

The significant difference between the mutant lines for most of the agronomic traits showed that EMS has successfully induced variations in most of the traits measured. This suggests that many genes controlling these traits were affected by the EMS treatment. Earlier studies have reported that EMS produces a large number point mutations in plants (Till *et al.*, 2003; Greene *et al.*, 2003; Till *et al.*, 2004).

Despite considerable level of variation observed for most the traits measured in this study, the level of variation was narrower than the ones that have been observed in

natural populations for traits such as first basal internode diameter, second basal internode diameter, first and second internode length, total culm length, and number of internodes (Assefa *et al.*, 1999; 2000; 2001). On the other hand, the value of agronomically important traits such as whole plant seed yield and whole plant biomass yields, and harvest index were higher in the present study than those reported by the above authors. This was expected because the mutation treatment was made on agronomically superior variety.

6.5 Conclusion

This study documented the successful induction of mutations for Al tolerance and several morpho-agronomic traits by using EMS. The screening procedures were efficient in identifying Al-tolerant lines. Induction of mutation by EMS may be utilized to develop Al tolerant varieties without sacrificing important agronomic traits, especially when used on popular varieties.

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CHAPTER 7

Development of a hydroponic facility as a phenotyping platform to assess for Al-tolerance in tef [*Eragrostis tef* (Zucc.) Trotter] using root growth measurements, and the haematoxylin staining technique

Abstract

Aluminium-toxicity contributes to 67% of crop production problem on acid soils of the world. Breeding crops for Al-tolerance is one strategy to cope with the Al-toxicity. Development of an appropriate phenotyping platform is a prerequisite to undertake Al-tolerance breeding. In Ethiopia, many soils can be classified as acid to severely acid. Tef is the most widely grown crop in Ethiopia, with a growing global popularity as source of a nutritious, gluten free flour. However, due to its restricted geographical region of production, tef has not been studied for tolerance to Al-toxicity. The objective of this study was to develop an appropriate phenotyping platform to screen for Al-tolerance in tef. Five levels of $AlK(SO_4)_2 .12H_2O$ (0, 150, 250,350, 450, 550 μM) were evaluated in order to select a moderately toxic level of Al to discriminate between sensitive and tolerant tef genotypes, assessed by measuring root growth. Further, the applicability of a haematoxylin staining method as a visual assay for Al-tolerance was assessed. There were highly significant differences ($P<0.001$) between the levels of Al-tolerance of the tef genotypes. The maximum differences in relative root length (RRL) (%) and root length (RL) (mm) between the most sensitive and the most tolerant tef genotypes were observed at the concentration of 150 μM. This concentration adequately discriminated between 28 tef genotypes with varied sensitivity to Al-toxicity. Using haematoxylin staining for the visual assessment of the roots of two sensitive and two tolerant genotypes treated with 0, 150 and 250 μM Al showed a differential staining reaction consistent with the root growth measurement methods. The hydroponic platform, combined with the root growth measurement method or haematoxylin staining can be accurately used to assess the levels of Al-tolerance in tef genotypes for genetic, breeding or physiological studies.

Key words: Aluminium toxicity, haematoxylin, hydroponics, phenotyping, tef

7.1 Introduction

Acid soils (pH < 5.5 in the surface layer) constitute 30% of the world's total ice free land. In Africa 22% land area has soil acidity problems (von Uexk¨ull and Mutert, 1995; Malcolm and Andrew, 2003). One of the effects of soil acidity is Al-toxicity, which affects at least 67% of crop production on acid soils of the world (Eswaran *et al.*, 1997). In addition to liming, worldwide, Al-tolerant genotypes of wheat, rice, maize, barley, sorghum and rye are used to cope with the problem of Al-toxicity (Pinto-Carnide and Guedes-Pinto, 1999; Hede *et al.*, 2001; Paterniani and Furlani, 2002; Portaluppi *et al.*, 2010).

Development of Al-tolerant crop genotypes requires various screening methods under field or controlled condition. Screening under hydroponics condition using toxic Al concentrations is widely used technique (Rao *et al.*, 1993; Hede *et al.*, 2001; Deborah and Tesfaye, 2003; Dharmendra *et al.*, 2011). This approach allows for direct access and non-destructive root measurements, which is not possible in field trials in soil. It also simplifies control over nutrient availability, pH, light conditions etc. (Carver and Ownby, 1995).

There are various formulations of nutrient solutions used in Al tolerance screening including the widely used Magnavaca's nutrient solution (Magnavaca *et al.*, 1987; Magalhaes *et al.*, 2004; Sasaki *et al.*, 2004; Magalhaes *et al.*, 2007). Recently, Famoso *et al.* (2010) modified Magnavaca's nutrient solution in order to closely mimic the low-ionic-strength and Al activity in acid soils. This formulation reduces precipitation of Al ions and increases the availability of important nutrients. But since different crop species have varied sensitivity to toxic concentration of Al, experimental determination of an appropriate level of Al is necessary for crop species not previously studied for their Al-tolerance (Hede *et al.*, 2001).

Under hydroponic culture, root growth measurement and haematoxylin staining reactions are widely used assay methods for Al-tolerance. Relative root tolerance index (RTI), which is computed as the ratio of root growth under toxic levels of Al to root growth without Al, is widely used to characterize the tolerance of crop species and genotypes to Al ions (Rao *et al.*, 1993; Hede *et al.*, 2001; Hede *et al.*, 2002).

Haematoxylin staining for the visual detection of tolerance to Al-toxicity in crops was first reported by Polle *et al.* (1978). Al-tolerant crop genotypes exclude phytotoxic Al-

ions from their root system by exudation of organic acids that complex with the Al ion in the rhizosphere and prevent their entry into the root system (Kochian *et al.*, 2005; Miyasaka *et al.*, 2007). Since sensitive genotypes lack this mechanism toxic Al ions easily enter the root tips, attach to nuclear and cytoplasmic targets and thereafter affect cell division and cell elongation in the transition region of the root apex (Miyasaka *et al.*, 2007) . In Al-tolerance assay, the Al already attached to nuclear and cytoplasmic targets serves as a mordant by atracting to these targets the negatively charged haematein of haematoxylin resulting in the development of a purple-blue colour dye that indicates presence of Al (Gill *et al.*, 1974; Polle *et al.*, 1978a; Kiernan, 2010).

Tef *[Eragrostis tef (Zucc.)]* is the most important cereal crop grown in Ethiopia. It is also a prospective global crop as a gluten free cereal and health food. The poor response of tef genotypes to fertilizer application on acid soils is one of the major constraints affecting tef production in Ethiopia (Mamo and Killham, 1987). Given the wide genetic diversity and agro-ecological adaptation of the crop, it may be possible to breed tef genotypes with Al- tolerance. No prior research work has been done on breeding for tolerance to Al-toxicity in tef. Development of a practical phenotyping platform such as the use of hydroponics technique combined with efficient assessment methods are a prerequisite to undertake breeding activities for Al-tolerance in tef. The aim of this study was threefold: To develop a hydroponics system as a phenotyping platform to test for Al-tolerance in tef genotypes; to determine an appropriate concentration of Al to evaluate Al-tolerance in tef; and to appraise the use of haematoxylin staining for visual assessment of Al-tolerance in tef seedlings.

7.2 Materials and methods

7.2.1 Determination of the optimum Al concentration and evaluation of selected tef genotypes for Al-tolerance using the selected concentration

7.2.1.1 Genetic stock

Two tef genotypes Acc#55185 and *Holeta Key*, were previously classified in trials as relatively tolerant and sensitive, respectively. These were used to identify the optimal Al concentration for Al-tolerance screening in tef. Another twenty eight tef genotypes were then evaluated for their Al-tolerance using the selected concentration of Al.

7.2.1.2 Experimental setup

Five Al concentrations were tested (0, 150, 250, 350, 450 and 550 $AlK(SO_4)_2 .12H_2O$ µM). These levels cover the range that is optimal when screening other cereals such as sorghum, maize, wheat and rice. A randomized complete block design (RCBD) with four replications and 7 plants per replication was used.

7.2.1.3 Plant growth condition

One hundred seeds per each variety were surface sterilized with 1% commercial bleach (sodium hypochlorite) for five minutes, the rinsed five times with sterile water. Then the seeds were placed in a sterilized glass Petri dish (100 x 15 mm) on Whatman filter paper wetted with sterile water. Germination took place over 24 hours under dark conditions at a temperature of 30^oC.

A recipe for a modified Magnavaca's nutrient solution were kindly provided by Dr Jon E. Shaff from Cornell University (Table 7.1). The nutrient solution was then supplemented with one of the six Al concentrations, 0, 150, 250, 350, 450, and 550 µm $AlK(SO_4)_2 .12H_2O$ µM. The pH of the treatment solutions was adjusted to 4.0 with 0.1 N HCl and/or 0.1 N KOH after Al was added. The pH of the control treatment was adjusted to 5.8 by using KOH.

After 24 hours, 7 uniformly germinated seedlings were selected and placed in holed Eppendorf tubes supported by acid washed silica sand (0.25mm-2.5mm) for each variety and Al treatment combination. The tubes were inserted to neoprene foam to float on the nutrient solution and were aerated with aquarium pump [Hydrofarm AAPA45L Active Aqua (www.hydrofarm.com)] fitted to infusion tubes clipped to the bottom of 4.5L plastic tubs by plastic tension clips (Figure 7.1). The pressure was regulated by the spigot of the air divider attached to the pump and the roller clamp of the infusion set to uniformly aerate the hydroponic solution in each in tub. The seedlings were exposed to 3600 lux cool white florescent lamp (16/8 light and dark hours) and were treated to Al ions for 4 days. The pH was continuously monitored. After the second day 50% of the hydroponic solution was changed.

Twenty eight tef genotypes were evaluated for their Al-tolerance under non replicated conditions using the same procedures and growth conditions but, using only one Al concentration.

7.2.1.4 Data collection and analysis

After 4 days in their respective Al solutions, the primary root length (mm) and shoot length (mm) of each plant were recorded. Total number and length (mm) of secondary roots and total number of dead plants were also recorded.

The mean primary root length was used to compute relative tolerance length. Relative tolerance length (RTL) (%) = (Value with Al /Value without Al) x 100.

Analysis of variance and a single degree of freedom contrast, and descriptive statistics, were computed using GenStat Statistical software Ver. 14 (GenStat., 2009). Excel 2013 was used to construct the graphs.

7.2.2 Assessment of haematoxylin staining

7.2.2.1 Genetic stock

The *Eragrostis tef* genotypes: *Holeta Key* and *Banja* local (an Al-tolerant landrace), and *Eragrostis pilosa* (L.*)* P. Beauv. (Acc-30-5) and *Eragrostis curvula* (Schrad.) Nees var. Ermelo were used to assess the reaction of the plants to haematoxylin staining after exposure to Al.

7.2.2.2 Experimental procedure

The seeds of the test genotypes were germinated following the procedure described above. The Petri dishes were kept in a growth chamber at 25^{o}C for 36 hours in the dark, and then 7 uniformly germinated seedlings were selected and placed in holed Eppendorf tubes to grow as described above. The hydroponic solution was supplemented with one of three doses of Al (0, 150, 250 µm $AlK(SO_4)_2.12H_2O$ µM). The pH of the Al solutions and the control were adjusted as indicated above.

After 24 hours, the seedlings were removed and rinsed with distilled water three times for 20 minutes with slow agitation to remove unbound Al. The roots were then immersed in a solution of 0.2% (w/v) haematoxylin and 0.02% (w/v) KIO_3 for 20 minutes with slow agitation (Cancado *et al.*, 1999). The stain was prepared a day before and was continuously stirred overnight to dissolve the haematoxylin (Delhaize *et al.*, 1993). After 20 minutes in the staining solution, the roots were removed and washed with distilled water for 30 minutes to remove excess stain. The roots were

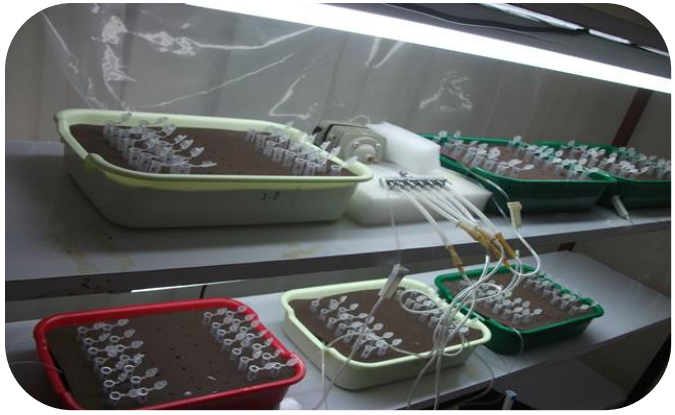
then rinsed with distilled water for 20 minutes with slow agitation and, kept wet and covered in a glass Petri dish until scoring for colour development. Finally, the staining pattern of the primary root, i.e., the tip 1.5 cm was viewed under a Nikon inverted microscope, and the degree of staining was scored on a 0-5 visual scale, where 0 was no stain and 5 was the maximal stain.

Table 7.1. Formulation of modified Magnavaca's nutrient solution used in the study

No	**Elements**	**Source Stock concentration**	**MW**	**g.L^{-1}**	**Volume (ml) of stock per 1 liter of treatment solution**
1)	**Ca**	**$Ca(NO_3)2.4H_2O$**	236	166.32	**5**
		NH_4NO_3	80	20.8208	
2)	**K**	**KCl**	74.6	8.5932	**5**
		K_2SO_4	174	20.328	
		KNO_3	101	11.3652	
3)	**Mg**	**$Mg(NO_3)_2.6H_2O$**	256.3	43.8592	**5**
4)	**P**	**KH_2PO_4**	136	1.232	**5**
5)	**Fe**	**$Fe(NO_3)_3.9H_2O$**	403.8	6.2216	**5**
		HEDTA	278.3	5.142368	
6)	**Micro nutrients**				
	Mn	**$MnCl_2.4H_2O$**	197.7	1.8018	**1**
	Bo	**H_3BO_3**	61.8	1.5708	
	Zn	**$ZnSO_4.7H_20$**	287.4	0.6776	
	Cu	**$CuSO_4.5H_20$**	249.5	0.154	
	Mo	**$Na_2.MoO_4.2H_2O$**	241.9	0.200	

Source: Jon E. Shaff, Cornell University (personal communication)

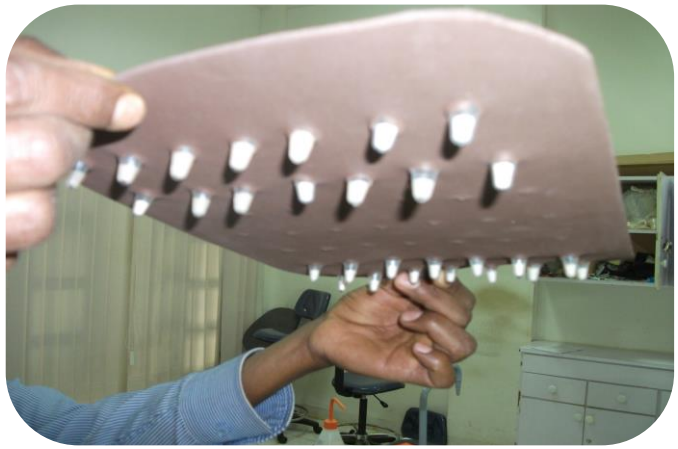
(A)-Areation system installation using infusion set



(D) Plants planted in tubes floating in foam

(C) A tef plant in an Eppendorf tube, supported by silica sand

(B) Aeration system working



(E) Silica sand support in holed tubes

Figure 7.1. Components of an indoor hydroponic system used to assess tef genotypes for Al-tolerance

7.3 Results

7.3.1 The hydroponics system as a phenotyping platform

The current indoor hydroponic phenotyping platform offered all the advantages of a controlled growth environment where the physical and the nutritional factors could be regulated. The physical environment including the temperature, and the relative humidity, and the light intensity, quality, and duration were controlled. As an indoor unit, the risk of contamination by dust particles was avoided. Algal development was also not noticed during the course of the experiment. A compact growth rack of 2 m height with inter-shelf spacing of 40 cm and width of 1.5 m has the potential to run many screening events at a time, all year round.

An infusion set (medical grade) was used for the aeration system. The self-sealing latex end was connected to the air divider which in turn was attached to the air pump. The spike and the drip chamber were easy to clip to the floor of the tub using plastic tension clips. The spike, the drip chamber and the pipe were kink resistant. In practice, they were efficient delivering adequate aeration from the bottom of the tub (Figure 7.1 A and B). The pressure was regulated by the spigot of an air divider attached to the pump and a roller clamp of the infusion set to uniformly aerate the tubs. Since the growth rack was compact with vertically arranged shelves, all the tubs were within the reach of the factory made infusion tubes with no need of air pipe extensions. The silica sand (0.25mm-2.5mm diameter) used to stabilize the seedlings also prevented the tiny germinated seeds of tef plants from escaping into the hydroponic tub through the hole at the base of the Eppendorf tubes (Figure 7.1 C and D).

The consistency in the pH records of the solution before and after addition of different concentrations of Al was used to avoid procedural faults and ensure the reproducibility of the protocols followed. The linear decline in root length and relative root length of the genotypes and the consistent increases of the root pruning effect of the Al ions associated with increases in Al concentration also confirmed the reliability of the procedures used.

7.3.2 Selection of Al concentration for tef screening

One-way analysis of variance for relative root length (RRL) (%) indicated highly significant differences ($P<0.001$) between the concentrations of Al for both the genotypes. The greatest reduction in RRL of 28% and 60% was observed between 0 and 150 µM $AlK(SO_4)_2 .12H_2O$ for the tef genotypes Acc#55185 and *Holleta Key*, respectively (Table 7.2). The reduction in RRL declined with increasing level of Al for both the genotypes. The difference in RRL, i.e., $T\text{-}S^{RRL}$ was at a maximum (32%) between the two genotypes when the Al concentration was 150 µM (Table 7.2).

Similarly, the one-way analysis of variance for actual root growth (mm) showed highly significant difference resulted from the differing Al levels. The difference in root length was at a maximum when the Al was increased from 0 to 150 µM for both the genotypes. The maximum difference for root length between the two genotypes was also observed at 150 µM of Al.

Table 7.2. Result of one-way analysis of variance for relative root length (RRL) (%) and Root length (Al+) (mm) of tolerant and sensitive tef materials

$AlK(SO_4)_2 .12H_2O$ (µM)	**RRL (%)**			**Rl (mm)**		
	Acc#55185 (T)	***Holleta Key* (S)**	**$T\text{-}S^{RRL}$**	**Acc#55185 (T)**	***Holleta Key* (S)**	**$T\text{-}S^{RL}$**
0	100.00a	100.00a	0	18.39a	14.75a	3.64
150	72.09b	40.24b	31.80	13.25b	5.79b	7.46
250	46.66c	20.54c	26.12	8.57c	2.93c	5.64
350	34.07d	17.49c	16.58	6.25d	2.57c	3.68
450	28.31de	14.96c	13.35	5.23de	2.14c	3.08
550	20.21e	13.65c	6.56	3.71e	1.96c	1.41
Mean	50.2	34.5	15.70	9.23	5.02	4.21
P (5%)	<.001	<.001		<.001	<.001	
F statistic	58.05	121.75		58.14	68.27	
LSD (0.05)	12.01	9.16		2.207	1.811	
CV (%)	15.9	17.6		15.9	23.9	

RRL-relative root length; Rl-root length; T-tolerant, S-sensitive; T-S-difference between the two.

The total number of dead plants was higher across all the Al levels for the sensitive variety. About 12 or 43% of the plants were dead for the sensitive variety at an Al level of 150 µM. At the same level of Al, no dead plants were recorded for the tolerant variety. The maximum difference between the tolerant and the sensitive for the number of dead plants was recorded at this level of Al (Figure 7.2).

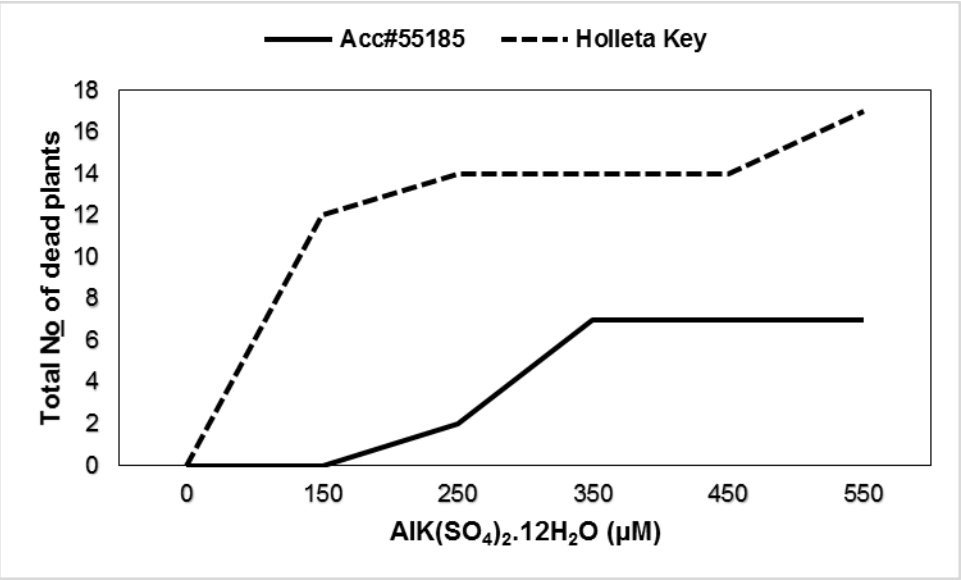


Figure 7.2 Total number of dead plants for the tolerant and sensitive genotypes across different concentrations of Al

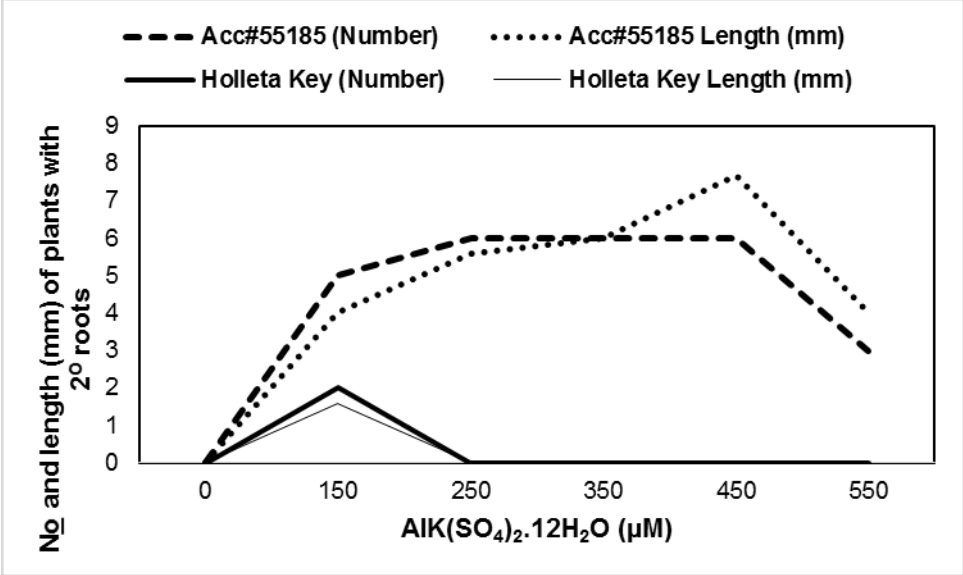


Figure 7.3 Number and total length of plants with secondary roots

The total number of plants with secondary roots and the total length of secondary roots was higher for the tolerant accession for all the Al levels. The maximum number of plants with secondary roots (6) and the maximum length (7.5 mm) of secondary roots was recorded for the tolerant accession at Al level of 450 µM. For the sensitive variety, the number of plants with secondary roots was at a maximum when the Al was 150 µM.

(Figure 7.3). Generally, it appeared that growth of secondary roots were initiated as a result of exposure of primary roots to Al as an adaptation mechanism.

7.3.3 Screening of selected tef genotypes for Al-tolerance

Differences were observed between tef genotypes screened for Al tolerance, both for relative root length (RRL) and root length (RL), when they were grown under hydroponic solution with 150 µM $AlK(SO_4)_2.12H_2O$ (Figure 7.4). The lowest RRL (%) was recorded for *E. pilosa* (Acc-30-5) and the highest was recorded for *E. curvula* var. Ermelo. The later had an RRL of over 100%, which was expected from prior experiments that had shown it to be a highly Al-tolerant species. Ten (36%) of the tested genotypes had RRL values of less than 50%. All the three parents of the mapping populations, i.e., *Key Murrie*, *DZ-01-2785* and *E. pilosa* (Acc 30-5) and nearly all the released genotypes, belonged to this group sensitive genotypes. Most of the accessions and the mutant lines had RRL value of over 50%. Among the released genotypes, *Mechare* and *Etsub* had relatively, higher level of Al-tolerance in that order. Overall, the wide variation observed showed that the selected concentration of Al was efficient in discriminating between the sensitive and tolerant genotypes. The contrast between the growth of Al-tolerant and sensitive genotypes is shown in Figure 7.5. The root pruning effects of Al were clearly demonstrated on Al-treated *E. pilosa* and *Holleta Key*.

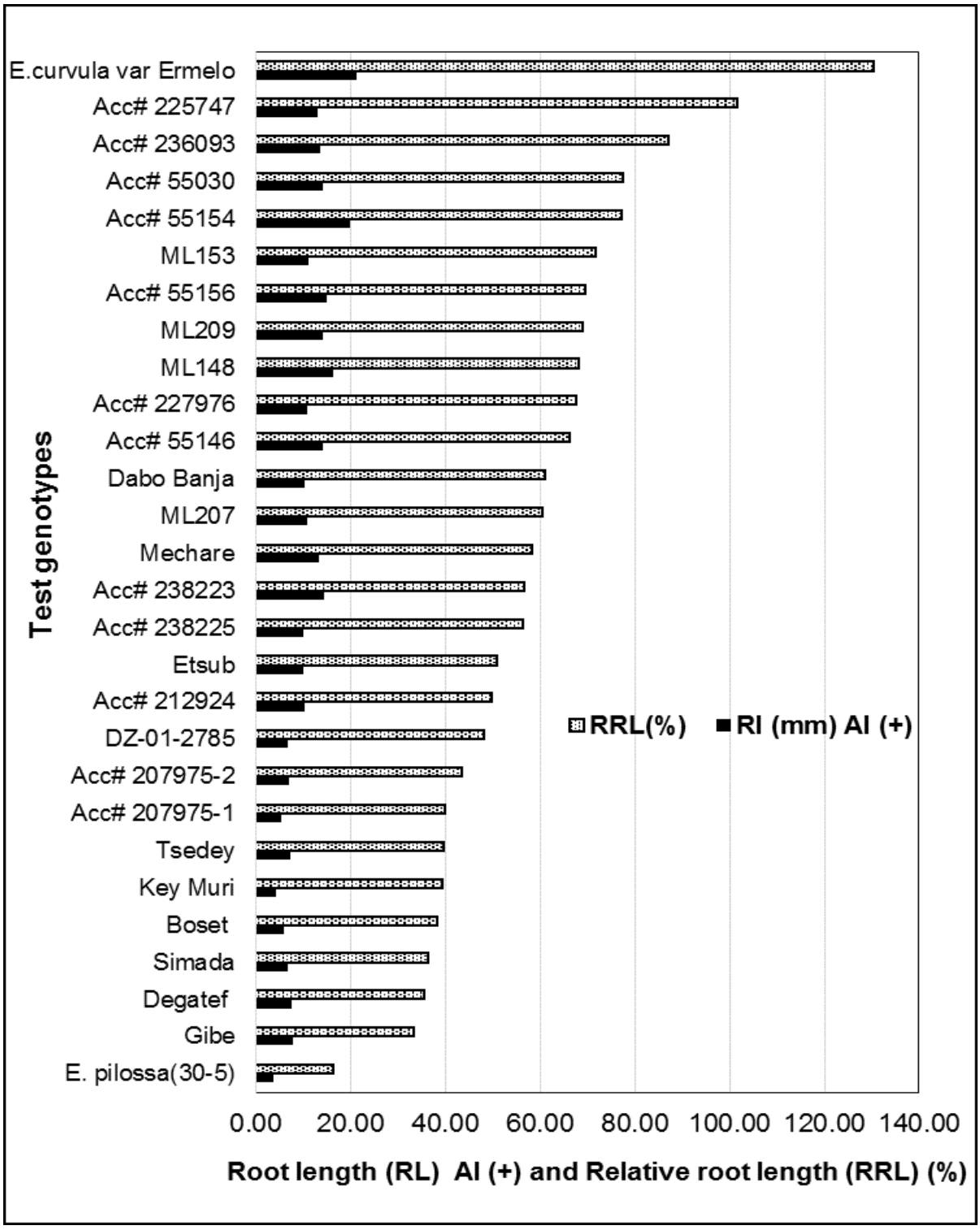
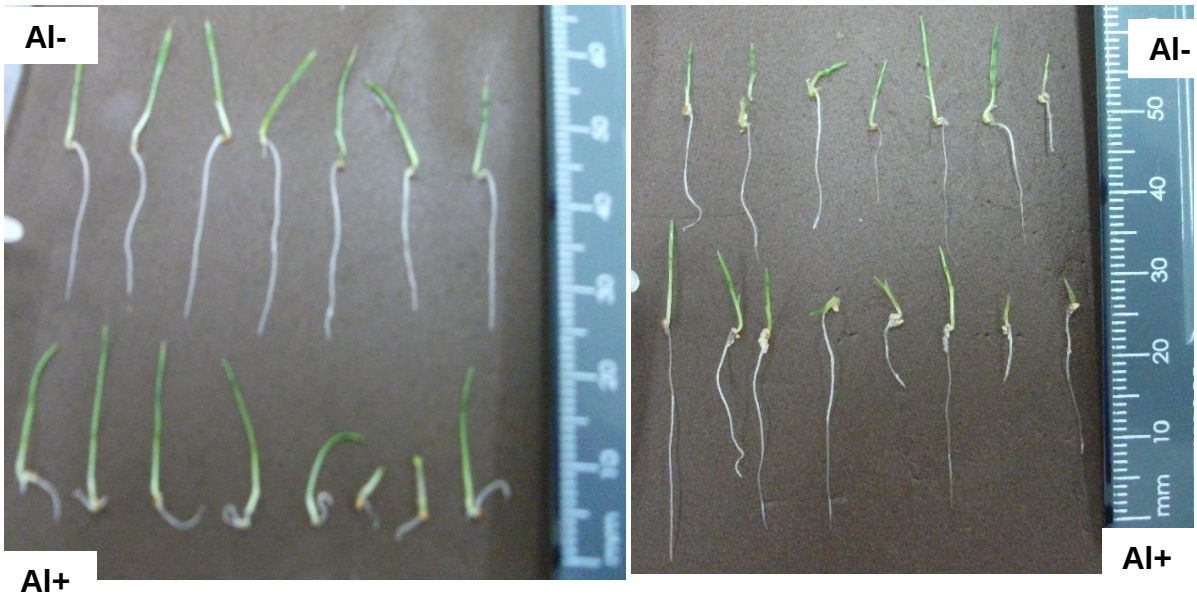


Figure 7.4 Root length (Rl) (mm) under 150 µM of $AlK(SO_4)_2.12H_2O$ and relative root length (%) of tef genotypes



E.pilosa (Acc 30-5) *E. curvula* var. Ermelo

Holleta Key (E. tef) Acc#55185 (*E. tef*)

Figure 7.5 Root growth of selected *E. tef*, *E. pilosa* and *E. curvula* genotypes with and with out 150 µM of $AlK(SO_4)_2.12H_2O$

7.3.4 Reaction of sensitive and tolerant *E. tef* genotypes to haematoxylin staining

A visual assessment of the reactions of two Al-tolerant genotypes *E. curvula* (var. Ermelo) and *E. tef (Dabo banja)* and two sensitive genotypes *E. pilosa* (Acc 30-5) and *E. tef (Holleta Key)* is presented in Figure 7.6. *E. curvula* showed no staining reaction across all the Al levels. The local Al-tolerant tef landrace, *Dabo banja*, showed a slightly purplish stain only at the highest level of Al. The two sensitive genotypes showed no staining at 0 Al and showed light purple and deep purple staining at concentrations 150 and 250 µM $AlK(SO_4)_2.12H_2O$, respectively. In both the sensitive materials, no staining reaction was observed on the outer most tip root part and the root cap of both light purple and deeply purple roots. In the rest of the roots, the tissues were uniformly stained with no uneven staining

Figure 7.6 Haematoxylin staining of the primary roots of Al-sensitive and tolerant genotypes of tef and related species treated with various concentrations of Al

7.4 Discussion

Hydroponic systems are often used as a phenotyping platform to assay Al tolerance using staining and root measurement techniques in several crop species (Tamas *et al.*, 2006; Narasimhamoorthy *et al.*, 2007; Famoso *et al.*, 2010; Portaluppi *et al.*, 2010). Compared to soil based techniques, hydroponics techniques of Al-tolerance screening allow for more stringent control over nutrient availability, Al-concentration, pH; aeration, light, temperature, and humidity. They also allow for the easy and non-destructive access to the root system, and facilitate the swift evaluation of large number of seedlings, yet require relatively little space (Carver and Ownby, 1995; Hede *et al.*, 2001; Raman and Gustafson, 2011).

The root staining and root measurement methods used to assay Al tolerance under hydroponic conditions have been correlated with the results of field experiments conducted on acid soils (Spehar, 1994; Baier *et al.*, 1995; Narasimhamoorthy *et al.*, 2007; Raman and Gustafson, 2011). In the present study, a hydroponic platform was successfully established with a high level of control over the growing conditions and is the first to be used to assess tef genotypes for Al-tolerance.

The modified Magnavaca's nutrient solution of Famoso *et al.* (2010) standardized for the screening of cereals was used in the present study. The five concentration of Al used in this study covered the concentrations of Al found to be most effective to screen for Al tolerance in sorghum (148 µM) (Magalhaes *et al.*, 2007), maize (222 µM) (Pineros *et al.*, 2005), and rice (540 µM) (Blamey *et al.*, 1991).

According to the Geochem-EZ chemical speciation model of Shaff *et al.* (2010), the free Al^{3+} activity of 148 µM, 222 µM and 540 µM $AlK(SO_4)_2.12H_2O$ in modified Magnavaca's nutrient solution was 27, 39 and 160 µM, respectively. In the present study, the selected Al level was 150 µM and this was equivalent to 148 µM $AlK(SO_4)_2.12H_2O$ or free Al^{3+} activity 27 µM determine to screen sorghum (Magalhaes *et al.*, 2007). The rate determined for tef in this study was higher than the 27 µM or free Al^{3+} activity of 8.75 µM found to be optimal for the screening of wheat by Sasaki *et al.* (2004).

The relative root length (RRL) and root length (RL) declined consistently with increases in the level of Al from 0-550 µM for both the sensitive and tolerant genotypes tested. Significant differences were observed in the sensitive variety in response to concentrations of 0, 150 and 250 µM. For the tolerant variety, significant differences were observed as a result of all changes in concentrations of Al. This suggests that a higher concentration of Al such as 250 and 350 µM could be used to differentiate between the levels of Al-tolerance of relatively Al tolerant genotypes. The difference between the two genotypes at a given Al concentration also declined as the concentration of Al was increased. The sensitive genotype was severely suppressed at the concentration of 550 µM Al for both Rl (mm) and RRL (%). Such a trend was also observed with wheat and sorghum in a previous study (Famoso *et al.*, 2010). Hence, 150 µM was selected due to the high level of variation observed for this concentration both within and between the categorized genotypes. The assessment of twenty eight tef genotypes covering the spectrum of Al-tolerance varied in their sensitivity to Al at a concentration of 150 µM, which showed that this concentration had the discriminatory power to differentiate between the extremely sensitive (*E. pilosa)* and the extremely tolerant species (*E. curvula*) species as well as the genotypes of *E. tef* with intermediate levels of Al-tolerance (Figures 7.4 and 7.5).

Hematoxylin is used in disease diagnosis and cytogenetic studies due to its powerful nuclear and chromatin staining properties. The staining mechanism is based on a haematein-mordant-cellular components interaction (Titford, 2005; Kiernan, 2010). The first application of haematoxylin staining for visual detection of tolerance to Al-toxicity in crops was reported by Polle *et al.* (1978) on wheat. The technique worked well on tef and accurately discriminated between Al-sensitive and Al-tolerant genotypes evaluated in this study.

In Al-sensitive genotypes, the negatively charged phosphate groups of DNA and the carboxyl group of proteins in cytoplasm are the main targets of toxic Al3+ ions (Matsumoto, 1991; Silva *et al.*, 2000; Kochian *et al.*, 2005; Miyasaka *et al.*, 2007). The Al atoms already attached to these nuclear and cytoplasmic targets of the root tips serve as a mordant by attaching these targets to negatively charged haematin,

resulting in the development of a purple-blue colour (Gill *et al.*, 1974; Polle *et al.*, 1978; Kiernan, 2010).

In the present study, the sensitive genotypes gave a positive reaction to the staining, whereas the tolerant genotypes did not Figures 7.5 and 7.6). This is in agreement with the use of the haematoxylin staining method to assess Al-tolerance in several cereals species including wheat, barley, sorghum and maize (Cancado *et al.*, 1999; Nawrot *et al.*, 2001; Anas and Yoshida, 2004; Stodart *et al.*, 2007).

In this study, the sensitive genotypes stained light purple at 150 µM and were deep purple at the concentration of 250 µM. The Al-tolerant local landrace, *Dabo banja*, showed slightly purple staining at the concentration of 250 µM. This result clearly shows that both the Al-concentration and the degree of host tolerance affect the intensity of colour development.

Most sensitive varieties accumulate more Al in their root and therefore their intensity of purple coloration is higher. Further, higher levels of Al have a tendency to overcome the inherent tolerance of the genotypes (Polle *et al.*, 1978; Cancado *et al.*, 1999). Hence, specific Al levels that give adequate level of contrast between the sensitive and tolerant genotypes are needed if the Al exclusion mechanism through exudation of organic acids is the only mechanism of tolerance and its genetic control is known. For maize and sorghum 222 µM Al has been used for haematoxylin staining (Cancado *et al.*, 1999; Anas and Yoshida, 2004).

In the sensitive genotypes that tested positive for the staining, the root cap and the outer most root tip did not stain at any concentration of Al. Aluminium has been reported to affect cell division and cell elongation in the transition region of the root apex (Miyasaka *et al.*, 2007). The differential reactions of Al-sensitive and Al-tolerant genotypes used in this study suggests that exclusion of Al from roots by organic acids may operate as a tolerance mechanism in tef. Nonetheless, since other tolerance mechanism that involve internal detoxification of Al after uptake by the root may stain positive for haematoxylin staining, positive staining does not necessarily indicate sensitivity. For instance, with rice the haematoxylin staining method does not discriminate between Al-sensitive and Al-tolerant genotypes in rice (Famoso *et al.*,

2011). In rice, which is the most Al-tolerant species of popular cereals, a number of internal detoxification mechanisms involving quantitative genes have been reported (Huang *et al.*, 2009; Xia *et al.*, 2010; Chen *et al.*, 2012; Huang *et al.*, 2012).

7.5 Conclusion

In the present study a highly controlled indoor hydroponic system was developed that allowed for efficient discrimination between Al-sensitive and Al-tolerant tef genotypes. An appropriate concentration of Al (150 µM) that adequately discriminated between Al-sensitive and Al-tolerant genotypes was also determined and was verified on 28 genotypes. The results were consistent with the results of pot experiments conducted earlier in this thesis. Haematoxylin staining was shown to provide an effective technique for the visual assessment of Al-sensitivity and Al-tolerance in tef for the first time.

In sets of genotypes evaluated in this study, Al-sensitive and Al-tolerant genotypes were consistently identified by both root growth measurement methods and haematoxylin staining methods. However, evaluations of many, diverse genotypes using haematoxylin staining compared with root measurements are needed before it would be safe to exclusively use this staining method as the only screening technique. Overall, the primary objective of this study was achieved, which was to develop a precise phenotyping platform to screen tef genotypes for Al-tolerance.

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A Thesis Overview of the Major Research Findings and Their Implications

Introduction

Soil acidity and Al-toxicity are major constraints affecting crop production in high rainfall areas of Ethiopia which are traditionally considered as ‘surplus production areas’ (high potential areas), as opposed to the moisture stressed areas. Nonetheless, breeding for crop tolerance for specific adaptations to low soil pH or Al-toxicity has not yet been prioritized for tef and other cereals. The deployment of Al-tolerant crop varieties is an important component of sustainable acid soil management strategies in the context of small-scale farmers. In the past, the lack of a tef breeding programmes for adaptations to marginal environments such as acid soils has contributed to a decline in the overall genetic gain from the national tef breeding programme in Ethiopia.

The present PhD research project was initiated with the overall objective of undertaking pre-breeding activities preceding the breeding of tolerance to Al-toxicity in tef. Accordingly, activities focused on: 1) The characterization of the acid soil production environments present in Ethiopia; 2) The evaluation of different sets of tef genetic resources, screening for tolerance to soil acidity and Al-toxicity; 3) An assessment of the potential to use EMS to induce enhanced Al-tolerance in tef; 4) the determination of genetic diversity in a number of tef accessions collected from areas affected by acid soils; 5) The development of a phenotyping platform for the screening of Al-tolerance in tef. This overview presents a summary of the major finding and their implications.

Soil acidity: importance, assessment of perceived causes and indicators, coping strategies and implications for the cereal-based, mixed-farming systems of north western Ethiopia

Major findings

- Soil erosion; competing use of local resources and poor nutrient recycling; abandoning of traditional fertility management practices; minimal and unbalanced use of external inputs; exclusive use of acid-forming inorganic fertilizers, DAP and urea, were identified by farmers as the causes of soil acidity.
- Soil erosion, soil acidity, the high cost of mineral fertilizers and lime, cash shortages, and a lack of seed of adaptable and high yielding crop varieties were identified as the top ranking constraints reducing tef production.
- Both the mitigation strategies promoted by the national extension service, and farmers' indigenous coping strategies, were constrained by various factors, and were not being implemented in practice.
- Species and varietal tolerance of soil acidity was found to be one of the major factors that influenced crop and variety choices by farmers.
- A decline in genetic diversity of once widespread crop species and landraces, and the rapid expansion of newly introduced, acid tolerant crops such as oat and triticale, were reported as clear indicators of the accelerating problems of soil acidity.
- The study sites were different in their edaphic and climatic conditions. Nonetheless, the pH (H_2O) of most of the soils of the study sites were all in a strongly acidic range (4.6–5.5). The soils of *Gashena Akayita* of Banja District were the most acidic of all, with high levels of exchangeable Al. Mn toxicity was also found to be a potential problem for the Districts of *Enguti* and *Enerata*.

Implications

- With the limited resources available to the farmers, sustainable management of acid soils and an improvement in the productivity of the farming systems seem unlikely, using the currently promoted management strategies. Hence, the

development of new technologies are needed that are compatible with the resources available to smallholders.

- Farmers' perceptions of soil acidity and their coping strategies are scientifically valid. Future research interventions need to capitalize on this knowledge if sustainable acid soil management is to be achieved. It was confirmed that the current top-down management approach of the extension service is not working, and is actually accelerating and widening the scale of the problem.
- The importance of acid soil tolerance in crop and variety selection, and the rapid expansion of acid soil tolerant crop species with relatively low market values, and the poor performance of the so called "improved varieties" in areas with acid soils, suggest that the distribution of new tef varieties that are highly tolerant of Al-toxicity and reliably produce higher yields in acid soils is the best technological option to offer to the farmers.
- Agro-ecological variations among the study sites suggest the need to address the full range of edaphic and climatic factors while breeding for tolerance to soil acidity and Al-toxicity.
- Monitoring changes in crop genetic diversity and undertaking collections of landraces may be needed in order to rescue local landraces of various crop species under replacement by more acid tolerant crops.

Response of selected tef [*Eragrostis tef* (Zucc.) Trotter] genotypes to soil acidity in pot and field experiments

Major findings

- The presence of genetic variation for Al-tolerance was demonstrated in tef for the first time.
- The relative value of various tolerance indices for Al-tolerance evaluation were assessed.
- Most of the Released Varieties were highly sensitive to soil acidity and Al-toxicity.
- Tolerant and sensitive varieties were identified for use in subsequent studies.

- The local check consistently outperformed the other varieties, both in the pot and field trials.
- A lack of adequate contrast for Al-tolerance was observed among the three parents of mapping population.
- When grown under unlimed condition, the grain yields of the 'improved' varieties, as well as the local check, were far below the national mean for grain yield by tef.
- Feedback from farmers on their selection criteria and priorities for tef varieties were gathered during an on-farm evaluation of the improved varieties by a test group of farmers.

Implications

- The existence of significant levels of genetic variation among the test genotypes suggests that it will be possible to successfully breed for Al-tolerance in tef.
- The high levels of sensitivity of the Released Varieties to Al-toxicity and their inferiority to the local landrace in both pot and field trials, suggests that attempts by the extension service to distribute these varieties to areas with acid soils is not viable, and will be detrimental to farmers growing these varieties.
- A lack of adequate contrast for Al-tolerance among the three parents of mapping population ruled out the possibility of using the mapping populations developed from these parents for molecular mapping of genes for tolerance to soil acidity and Al-toxicity.
- The consistent superiority of the local landrace, which is widely grown in the most acidic environments, indicates that there has been selection by farmers for tolerance to acid soils and of the acid tolerant landraces to the climatic conditions occurring in the areas with acid soils. This suggests that breeding for tolerance to acid soils must begin with a broad evaluation of tef genetic resources sourced from areas with acid soils.
- An extremely high level of Al-tolerance was demonstrated for the closely related species *E. curvula* var. Ermelo in this study. Further studies on this species are warranted. This species could be used to enhance Al-tolerance in tef through conventional or gene transfer approaches, in the long run.

Evaluating the genetic diversity of tef [*Eragrostis tef* (Zucc.) Trotter] accessions collected from sites in Ethiopia with acid soils, using simple sequence repeats (SSR) markers

Major findings

- The selected SSR markers were effective at discriminating between the tef genotypes examined.
- Analysis of molecular variance (AMOVA) showed highly significant differences ($P<0.001$) existed among and within populations.
- Despite the wide geographical separation of the collection sites, 88.5% of the acid soil accessions could be grouped into two clusters (Clusters II and III), while 90% of the Breeding Materials and Released Varieties could be grouped into Cluster I.
- Accessions from north western Ethiopia exhibited a significant level of variation for most of the genetic diversity parameters.
- The number of private alleles was significantly higher for tef varieties in the acid soil collections compared to the Released Varieties and the Breeding Materials.
- Pair wise estimates of genetic identity and gene flow showed higher values between Released Varieties and Breeding Materials.

Implications

- A high levels of genetic diversity was found in acid soil accessions, which offers plant breeders an opportunity to breed for acid tolerant and agronomically superior varieties of tef. This will help to improve food security and the livelihoods of small-scale farmers, and will also help to increase and preserve rare alleles that may confer acid tolerance under farmers' conditions.
- The lower number of private alleles in Released Varieties can be related to the negative impact of plant breeding in narrowing the gene pool.
- The current attempts to promote the use of released varieties of tef and other recently bred crop varieties in areas affected by acid soils, and the rapid

expansion of newly introduced, acid-tolerant crop species such as oat and triticale, put at risk the local landraces of tef. There is a clear need to collect a representative spectrum of accessions from acid soil environments.

- The result of this study suggests the need to include all acid soil affected regions, and maximize the within region sampling during germplasm collection activities.

Isolation and characterization of ethyl methane sulphonate (EMS) induced mutants of tef [*Eragrostis tef* (Zucc.) Trotter] for Al-tolerance and morpho-agronomic traits

Major findings

- This study is the first to report the successful artificial mutation of tef for enhanced Al-tolerance using EMS.
- The screening technique combined the use of a strongly acidic soil with an external application of a toxic concentration of an Al solution, and subsequently exposed the test plants to drought for 96 hours. This was successful in identifying mutant lines with enhanced Al-tolerance.
- There were significant differences ($P<0.001$) between mutant lines; between the mutant lines and the parent; and between mutant lines and the sensitive check. However, no significant difference was observed between the mutant lines and the tolerant check.
- Significant differences were also observed between the mutants for 16 of the 20 quantitative traits measured, confirming that EMS induced mutagenesis is a successful approach to creating a diversity of phenotypic expression in tef.

Implications

- EMS can be used to induce mutations in tef that include mutations for enhanced levels of Al-tolerance in Al-sensitive but popular tef varieties.
- The screening technique employed in this study can be used in similar studies that aim at the identification of Al-tolerant breeding materials in tef and other crops.

- The mutant lines with enhanced Al-tolerance identified in this study shall be evaluated for important agronomic traits and their adaptability to other edaphic and climatic conditions in targeted environments with acid soils.

Development of a hydroponic facility as a phenotyping platform to assess for Al-tolerance in tef [*Eragrostis tef* (Zucc.) Trotter] using root growth measurements, and the haematoxylin staining technique

Major findings

- A highly controlled, indoor hydroponic system was developed that provided characterization reliable facility for the accurate assessment of tef breeding materials for levels of Al-tolerance or sensitivity.
- A level of 150 µM $AlK(SO_4)_2.12H_2O$ was found to be an appropriate dosage in order to screen tef for Al-tolerance under hydroponics conditions.
- Haematoxylin staining was verified for the first time on tef as a visual assessment technique to identify Al-tolerance in the roots of tef plants..

Implications

- The novel hydroponic facility, and the root growth measurement methods tested on tef plants grown in the facility, can be used as a reliable phenotyping platform to launch a breeding programme to breed novel varieties of tef and other crops that will perform well in environments in Ethiopia with acid soils.
- In a range of tef germplasm screened in this study, the result of the haematoxylin staining was consistent with that of the root growth measurement method. However, a future evaluation of more tef breeding materials using haematoxylin staining and root measurements is needed to conclusively prove the validity of the haematoxylin staining method for tef evaluations.
- The superior genotypes evaluated in this study can be used as genetic stocks for future breeding programmes and genetic studies of Al-tolerance in tef.