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Investigation of Al-toxicity tolerance in tef (*Eragrostis tef)* under hydroponic system using root growth measurement and haematoxylin staining methods

Ermias Abate[1,2]*, Shimelis Hussein[2], Assefa Amelework[2,3], Jon E. Shaff[4], Mark Laing[2], Zerihun Tadele[5], Fentahun Mengistu[6]

[1]Amhara Regional Agricultural Research Institute, P.O.Box-527, Bahir Dar, Ethiopia
[2] School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal African Centre for Crop Improvement, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa
[3]Agricultural Research Council, Vegetable, Industrial and Medicinal Plants, Private Bag X293, Roodeplaat, Pretoria 0001, South Africa
[4] Robert W. Holley Centre of Agriculture and health, USDAARS, Cornell University, Ithaca, New York, USA
[5]University of Bern, Altenbergrain 21, 3013 Bern, Switzerland, [5]Institute of Biotechnology, Addis Ababa University, P.O. Box 32853, Addis Ababa, Ethiopia
[6]Sasakawa Africa Association/ Sasaakawa Global 2000 Ethiopia, P.O.Box 12771, Addis Ababa, Ethiopia

***Corresponding author: assefaa@arc.agric.za**

Abstract

Tef is the most widely grown and consumed crop in Ethiopia. The crop is currently gaining growing popularity worldwide as nutritious and gluten free cereal crop. Al-toxicity is one of the factors that limit expansion of the crop worldwide. The aims of this study were to adapt hydroponic system as a phenotyping platform for Al-tolerance studies in tef, to identify appropriate concentration of Al^{3+} for Al-tolerance screening in tef, and to appraise the use of haematoxylin staining for visual assessment of Al-tolerance in tef. A tolerant and a sensitive genotypes were used to identify appropriate Al concentration. The identified Al concentration was used to evaluate reaction of 28 tef genotypes to Al-toxicity. Root and shoot measurements under Al-treated and control solution were used to compute the relative tolerance indexes. Reaction of the tolerant and the sensitive genotypes to haematoxylin staining was assessed by recording intensity of colour development. The result indicate that, among the five levels of $AlK(SO_4)_2.12H_2O$ (0-550 µM), the 150 µM concentration was found to be an appropriate level to discriminate between sensitive and tolerant genotypes of tef. This concentration adequately differentiated 28 tef genotypes with varied sensitivity to Al-toxicity. The visual assessment of selected tolerant and sensitive genotypes treated with 0, 150 and 250 µM $AlK(SO_4)_2 .12H_2O$ showed a differential reaction to haematoxylin staining consistent with the root growth measurement methods. The tolerant *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 (*E. Pilossa* and Holeta Key), however, showed deep purple staining at concentrations of 150 and 250 µM $AlK(SO_4)_2.12H_2O$. To the best of the authors' knowledge, this study is the first to report the use of root measurement and root staining methods in evaluation of tef for tolerance to Al-toxicity using hydroponics platform.

Key words: Aluminium toxicity, haematoxylin, hydroponics, root growth, tef.
Abbreviation: CSA Central Statistics Agency; CV_ Coefficient of Variation; LSD_ Least Significant Difference; RCBD_ Randomized complete block design; RRL_ Relative root length; RTI_ Relative Root Tolerance Index; RL_ Root Length; Zn_ Zinc.

Introduction

Tef [*Eragrostis tef* (Zucc.) Trotter] is the most widely cultivated and economically important cereal crop in Ethiopia (CSA, 2020). It is also a prospective global crop as a gluten free cereal and health food. However, tef is the least yielding crop among all cereals crops grown in Ethiopia. Al-toxicity is one of the factors that hamper productivity and expansion of the in Ethiopia.

Acid soils (pH < 5.5 in the surface layer) constitute 30% of the world's total ice-free land. About twenty-two percent of Africa's total land area is affected by soil acidity (von Uexk¨ull

and Mutert, 1995, Malcolm and Andrew, 2003). Worldwide, about 67% of crop production constraint on acid soils is associated to Al-toxicity (Eswaran et al., 1997). In addition to liming, Al-tolerant varieties are widely used to produce globally important staple cereals on acid soils with Al-toxicity problem (Hede et al., 2001; Paterniani and Furlani, 2002; Pinto-Carnide and Guedes-Pinto, 1999, Portaluppi et al., 2010). Tef has not been studied for its tolerance to Al-toxicity. Availability of wide genetic diversity in tef and its broad agro-ecological adaptation, offers the opportunity to develop tef materials that can tolerate Al-toxicity.

Morphological, physiological and biochemical traits are widely used techniques to identify Al-toxicity tolerant varieties under field and greenhouse screening (Carvalho et al., 2016). Field screening for Al tolerance offers advantage identifying varieties that are not only tolerant to Al-toxicity but also adapted to the complex growing environment of the target production areas (Yang et al., 2013). In practice, however, it is difficult to expose genotypes to uniform Al concentration and identify truly tolerant varieties under field condition (Rao et al., 2016). Thus, development of appropriate and high-throughput phenotyping platform and efficient assessment methods appropriate for early stage screening of varieties is a prerequisite to identify Al-toxicity tolerant crop varieties that will be eventually tested under field condition. Screening under hydroponics condition using toxic Al concentrations is the most efficient, suitable and widely used technique (Deborah and Tesfaye, 2003; Dharmendra et al., 2011; Hede et al., 2001; Rao et al., 1993). This approach simplifies control over nutrient availability, pH, light conditions and allows easy access and non-destructive root measurements compared to phenotyping under pot and field condition (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; Magalhaes et al., 2007; Sasaki et al., 2004). Recently, Famoso et al. (2010) modified the Magnavaca's nutrient solution so that it closely mimics the low ionic strength and Al activity in acid soils and named it 'modified the Magnavaca's nutrient solution'. Further, this formulation has preferred features of reduced precipitation of Al ions and increased availability of important nutrients compared to other formulations. Since different crop species have varied sensitivity to toxic concentration of Al, determination of an appropriate level of Al is necessary to screen genotypes and crop species for Al-toxicity tolerance (Hede et al., 2001).

Root pruning is the major and distinct effect of Al^{3+} in sensitive plants. Thus, root growth measurement methods are widely used to assay Al-tolerance under hydroponic system. Relative root tolerance index (RTI), which is computed as the ratio of root growth under toxic levels of Al^{3+} to root growth without Al^{3+} (control), and measurement of root growth under toxic concentration are most often used parameters to characterize the tolerance of crops to Al^{3+} (Hede et al., 2001; Hede et al., 2002; Rao et al., 1993).

Haematoxylin staining is a visual detection method used to assay cereal crops and grass species for Al-tolerance under hydroponic platform. Al-tolerant crop varieties exclude phytotoxic Al^{3+} from their root system by exudation of organic acids to the rhizosphere (Kochian et al., 2005; Miyasaka et al., 2007). Al sensitive genotypes, on the other hand, lack this mechanism and toxic Al^{3+} easily enter the root tips, attach to nuclear and cytoplasm and affect cell division and cell elongation in the transition region of the root apex (Miyasaka et al., 2007). In Al-tolerance assay, the Al^{3+} ions attached to celluar targets can serve as a mordant that binds the negatively charged haematein of haematoxylin resulting in the development of a purple-blue colour dye (Gill et al., 1974; Kiernan, 2010; Polle et al., 1978). The aims of this study were to adapt a hydroponic system as a phenotyping platform for Al-toxicity tolerance in tef, to identify appropriate concentration of Al^{3+} for tef Al-toxicity tolerance screening, and to appraise the use of haematoxylin staining for visual assessment of Al-tolerance in tef seedlings.

Results

The hydroponics system as a phenotyping platform

The current 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 temperature, relative humidity, and light intensity, quality, and duration were controlled. Being an indoor unit, the risk of contamination by foreign 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 x 1.5 m has the potential of running many screening events at a time and 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 in delivering adequate aeration from the bottom of the tub (Figure 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 (0.25 - 2.5 mm 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 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. 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 procedure used.

Determination 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^{3+} 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 3). The reduction in RRL declined with increasing level of Al for both the genotypes. The difference in RRL, T-S^{RRL} was at a maximum (32%) between the two genotypes when the Al concentration was 150 µM (Table 3).

Similarly, the one-way analysis of variance for actual root growth (mm) showed highly significant difference. 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.

The total number of dead plants was higher across all the Al levels for the sensitive variety. The idea behind using dead plants information was to choice of the appropriate rate that discriminates the tolerant from sensitive genotypes. About 12 or 43% of the plants were dead for the sensitive variety at an Al^{3+} level of 150 µM. At the same level of Al^{3+}, 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^{3+} (Figure 2).

The total number of plants with secondary roots and the total length of secondary roots were higher for the tolerant accession for all the Al^{3+}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^{3+} was 150 µM. (Figure 3). Generally, it appeared that growth of secondary roots was initiated as a result of exposure of primary roots to Al as an adaptation mechanism.

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 4). The lowest RRL was recorded for *E. pilosa* (Acc-30-5) and the highest was recorded for *E. curvula* (Ermelo). The later had an RRL of over 100%, which was expected from a highly Al-tolerant species. Thirty-six percent of the tested genotypes, which include the three parents of the mapping populations (*Key Murrie*, *DZ-01-2785* and Acc 30-5) and nearly all the released varieties, had RRL values of less than 50%. Most of the landraces and the mutant lines revealed RRL values of over 50%. Among the released genotypes, *Mechare* and *Etsub* had relatively higher level of Al-tolerance in that order. The overall wide variation in RRL observed in this study indicated that the selected concentration of Al was sufficient in discriminating the genotypes as sensitive and tolerant. The contrast between the growth of Al-tolerant and sensitive genotypes is shown in Figure 4. The root pruning effect of Al^{3+} was clearly demonstrated on Al-treated *E. pilosa* and *Holleta Key* (Figure 5).

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 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 the Al-sensitive genotypes, although uniform and evenly deep staining patterns were observed, no staining was observed on the outermost root part and the root cap.

Discussion

Hydroponic system is commonly used for phenotyping of Al tolerance using root growth measurements and staining techniques in several crop species (Famoso et al., 2010; Narasimhamoorthy et al., 2007; Portaluppi et al., 2010; Tamas et al., 2006). The hydroponics techniques of Al-tolerance phenotyping allows for more stringent control over pH, aeration, light, temperature, and nutrient availability, Al-concentration, and humidity compared to field screening techniques. These techniques also allow for the easy and non-destructive sampling of the root system, and facilitate the swift evaluation of large number of seedlings in a relatively small 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 (Baier et al., 1995; Narasimhamoorthy et al., 2007; Raman and Gustafson, 2011; Spehar, 1994). 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.

Haematoxylin staining techniques are the most widely used histochemical assay for disease diagnosis and cytogenetic studies (Titford, 2005). The staining mechanism is based on a haematein-mordant-cellular components interaction (Kiernan, 2010; Titford, 2005). Polle et al. (1978) was the first to employ haematoxylin staining for visual detection of Al-tolerance 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 Al^{3+} (Kochian et al., 2005, Matsumoto, 1991, Miyasaka et al., 2007, Silva et al., 2000). 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, Kiernan; 2010, Polle et al., 1978).

In the present study, the sensitive genotypes gave a positive reaction to the staining, as opposed to the tolerant genotypes. This is in agreement with the use of the haematoxylin staining method to assess Al-toxicity tolerance in several cereals species including wheat, barley, sorghum and maize (Anas and Yoshida, 2004; Cancado et al., 1999; Nawrot et al., 2001, Stodart et al., 2007). In this study, the sensitive genotypes stained light purple at 150 µM and were deep blue at the concentration of 250 µM. In this study, in the sensitive genotypes, the lack of stains in the outermost layers of the roots and the root cap, suggesting Al^{3+} has been reported to affect cell division and cell elongation in the transition region

Table 1. List of genotypes used in the screening.

Genotypes	Species	Status
Dabo Banja	*Eragrostis tef*	Local check
ML207	*Eragrostis tef*	Mutant line
ML148	*Eragrostis tef*	Mutant line
ML209	*Eragrostis tef*	Mutant line
ML153	*Eragrostis tef*	Mutant line
DZ-01-2785	*Eragrostis tef*	Parent for mapping population
Acc# 207975-1	*Eragrostis tef*	Purified accession
Acc# 207975-2	*Eragrostis tef*	Purified accession
Acc# 212924	*Eragrostis tef*	Purified accession
Acc# 238225	*Eragrostis tef*	Purified accession
Acc# 238223	*Eragrostis tef*	Purified accession
Acc# 55146	*Eragrostis tef*	Purified accession
Acc# 227976	*Eragrostis tef*	Purified accession
Acc# 55156	*Eragrostis tef*	Purified accession
Acc# 55154	*Eragrostis tef*	Purified accession
Acc# 55030	*Eragrostis tef*	Purified accession
Acc# 236093	*Eragrostis tef*	Purified accession
Acc# 225747	*Eragrostis tef*	Purified accession
Gibe	*Eragrostis tef*	Released variety
Degatef	*Eragrostis tef*	Released variety
Simada	*Eragrostis tef*	Released variety
Boset	*Eragrostis tef*	Released variety
Key Muri	*Eragrostis tef*	Released variety
Tsedey	*Eragrostis tef*	Released variety
Etsub	*Eragrostis tef*	Released variety
Mechare	*Eragrostis tef*	Released variety
Acc-30-5	*Eragrostis pilosa*	Related species
Ermelo	*Eragrostis curvula*	Related species

Eragrostis curvula (Schrad.) Nees var. Ermelo, *Eragrostis pilosa* (L.*)* P. Beauv. (Acc-30-5).

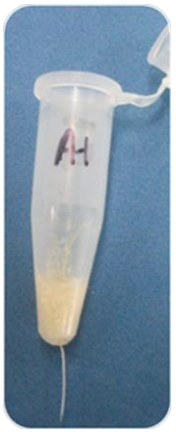
Table 2. Formulation of modified Magnavaca's nutrient solution used in the study.

No	Elements	Source stock concentration	MW	$g.L^{-1}$	Volume (ml) of stock per 1 liter of treatment solution
1	Ca	$Ca(NO_3)_2\ 4H_2O$	236.0	166.3200	5.0
		NH_4NO_3	80.0	20.8208	
2	K	KCl	74.6	8.5932	5.0
		K_2SO_4	174	20.328	
		KNO_3	101	11.3652	
3	Mg	$Mg(NO_3)_2\ 6H_2O$	256.3	43.8592	5.0
4	P	KH_2PO_4	136.0	1.2320	5.0
5	Fe	$Fe(NO_3)_3\ 9H_2O$	403.8	6.2216	5.0
		HEDTA	278.3	5.1424	
6	Micro nutrients				
	Mn	$MnCl_2.4H_2O$	197.7	1.8018	1.0
	B	H_3BO_3	61.8	1.5708	
	Zn	$ZnSO_4.7H_20$	287.4	0.6776	
	Cu	$CuSO_4.5H_20$	249.5	0.1540	
	Mo	$Na_2.MoO_4.2H_2O$	241.9	0.2000	

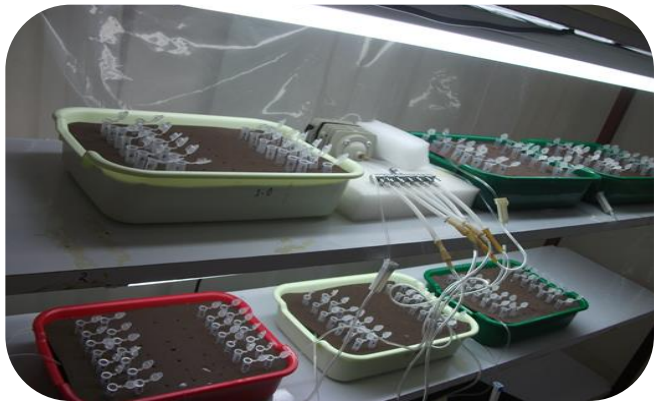
Source: Jon E. Shaff (Cornell University).

(A)-Areation system installation using infusion set

(B) Aeration system working



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



(D) Plants planted in tubes floating in foam

(E) Silica sand support in holed tubes

Figure 1. Components of an indoor hydroponic system used to assess Al-tolerance in tef genotypes.

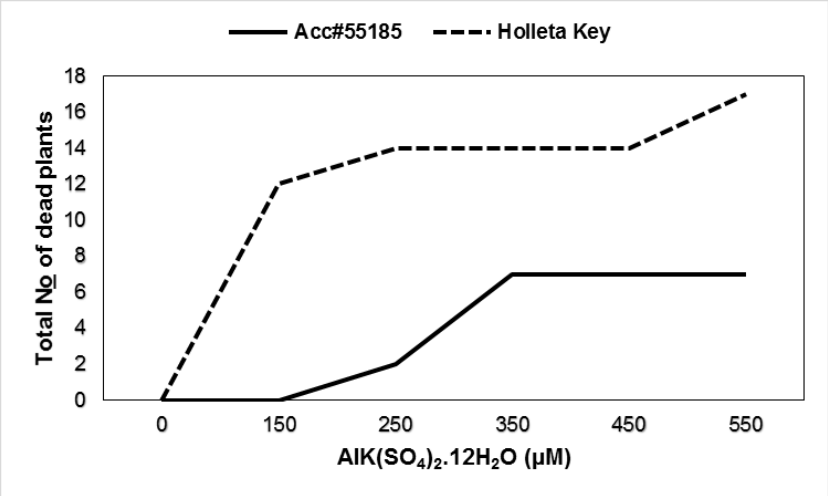


Figure 2. Total numbers of dead plants for the tolerant and sensitive genotypes across different Al concentrations.

Table 3. Result of one-way analysis of variance for relative root length (RRL)(%) and Root length (RL) (mm) of tolerant and sensitive tef materials.

$AlK(SO_4)_2.12H_2O$ (µM)	RRL (%)			RL (mm)		
	Acc#55185 (T)	*Holleta Key* (S)	T-S^{RRL}	Acc#55185 (T)	*Holleta Key* (S)	T-S^{RL}
0	100.00a	100.00a	0.00	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.						

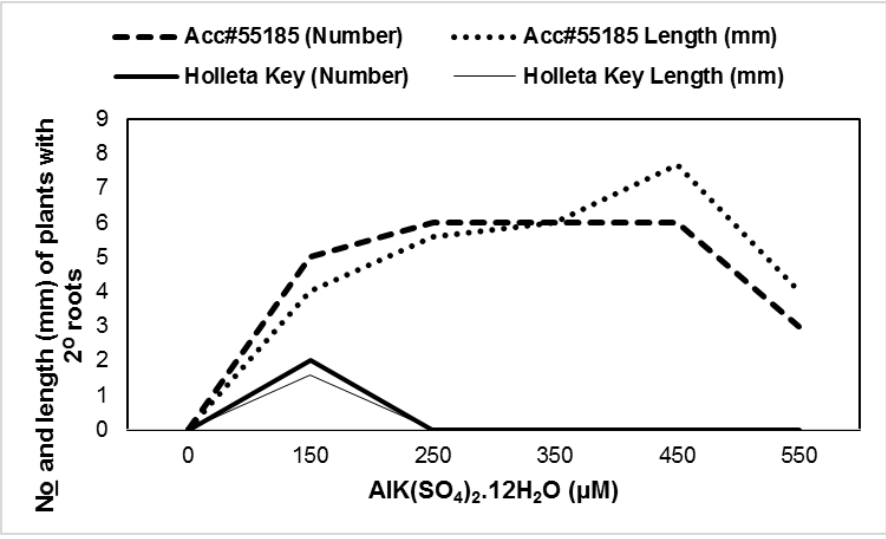


Figure 3. Number and total length of plants with secondary roots.

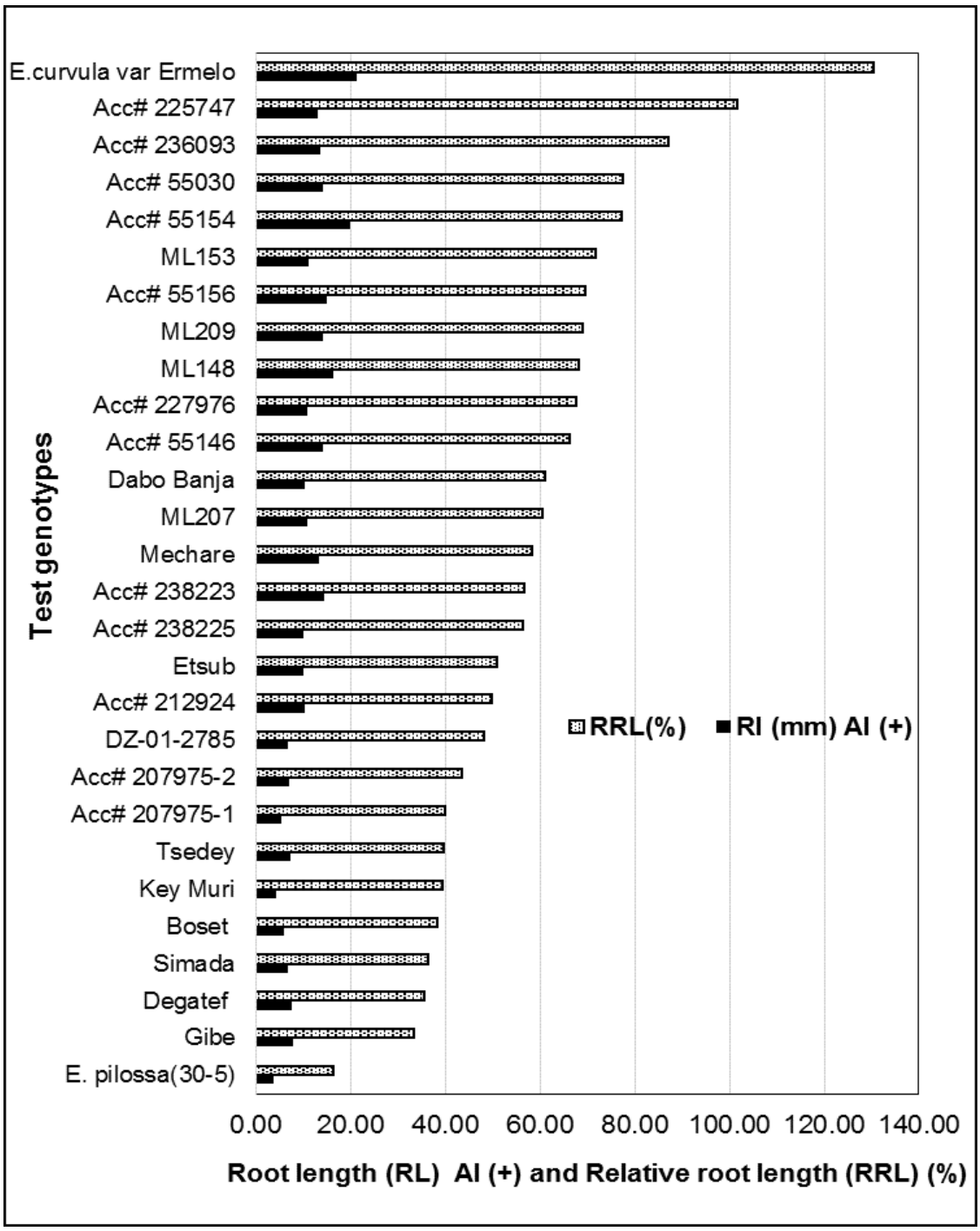
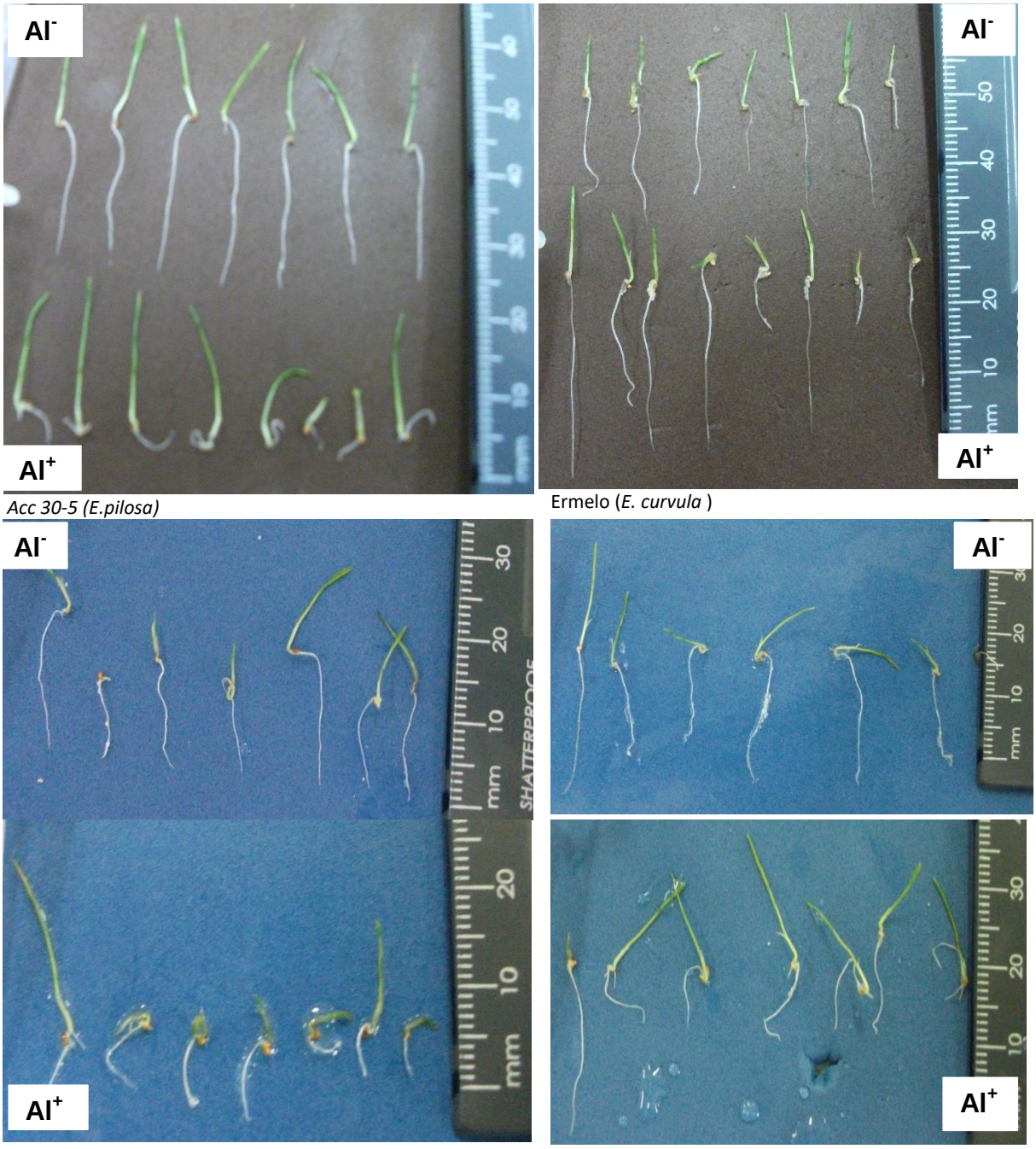


Figure 4. Root length (RL) (mm) and relative root length (RRL) of tef genotypes tested under 150 µM of $AlK(SO_4)_2.12H_2O$ concentration.



Acc 30-5 (E.pilosa)

Ermelo (*E. curvula*)

Holleta Key (E. tef)

Acc#55185 (*E. tef*)

Figure 5. Root growth of selected *E. tef*, *E. pilosa* and *E. curvula* genotypes with and with out 150 µM of $AlK(SO_4)_2.12H_2O$.

Figure 6 Haematoxylin staining of the primary roots of Al-sensitive and Al-tolerant genotypes of tef and related species treated with various concentrations of Al.

of the root apex (Miyasaka et al., 2007). On the other hand, the observed deep blue staining patters that extends into epidermal and cortical tissues suggests that these tissues may be the possible Al binding sites in the root system.

The absence of stains in Ermelo *(E. curvula)* at 250 µM $AlK(SO_4)_2.12H_2O$ concentration indicating that this variety is very tolernat (Ermias et al., 2013, Miles and Villiers, 1989). However, the slight purple stains observed on the Al-tolerant local landrace, *Dabo banja*, at the same concentration clearly shows that both the Al-concentration and the degree of host tolerance affect the intensity of colour development. The differential reactions of Al-sensitive and Al-tolerant genotypes used in this study, suggests that exclusion of Al^{3+} 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 (Chen et al., 2012; Huang et al., 2009; Huang et al., 2012; Xia et al., 2010).

Most sensitive varieties accumulate more Al^{3+} in their root and therefore their intensity of purple coloration is higher. Further, higher levels of Al^{3+} have a tendency to overcome the inherent tolerance of the genotypes (Cancado et al., 1999, Chlipala et al., 2020; Polle et al., 1978). Hence, specific Al^{3+} 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 (Anas and Yoshida, 2004; Cancado et al., 1999).

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^{3+} used in this study covered the concentrations of Al^{3+} found to be most effective to screen for Al-toxicity 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 Al3+ activity of 148 µM, 222 µM and 540 µM AlK(SO4)2.12H2O in modified Magnavaca's nutrient solution was 27, 39 and 160 µM, respectively. In the present study, the selected Al concentration level was 150 µM and this was equivalent to 148 µM AlK(SO4)2.12H2O or free Al3+ 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 and root length declined consistently with an increase in the level of Al^{3+} from 0-550 µM in both the sensitive and tolerant genotypes. Significant differences were observed in RRL and RL in the sensitive genotypes in response to concentrations of 0, 150 and 250 µM. The significant differences observed in the tolerant variety were associated with the changes in concentrations of Al. This suggests that the higher concentrations of Al^{3+} such as 250 and 350 µM can be used to differentiate between the levels of Al toxicity tolerance among tolerant genotypes. The difference between the two genotypes at a given Al^{3+} concentration also declined as the concentration of Al increased. The root development (RRL and RL) in the sensitive genotype was severely suppressed at the concentration of 550 µM Al. This trend was also observed with wheat and sorghum in a previous study (Famoso et al., 2010). Hence, 150 µM was selected as an optimum Al level for Al toxicity study because at this concentration, high level of variation was observed within and between the categorized genotypes. The assessment of twenty-eight tef genotypes covering a wide spectrum of Al-tolerance varied in their sensitivity to Al at a concentration of 150 µM. This showed that this concentration had the discriminatory power to differentiate extremely sensitive (*E. pilosa)* from extremely tolerant (*E. curvula*) species as well as the genotypes of *E. tef* with intermediate levels of Al-tolerance.

Materials and methods

Genetic stock

Preliminary study was conducted using two tef genotypes, Acc#55185 and *Holeta Key*, representing tolerant and sensitive genotypes (Ermias et al., 2015, 2017), respectively, to identify the optimal Al concentration for Al-toxicity tolerance screening. The identified concentration was used to evaluate twenty-eight tef genotypes consisting of released varieties, related species, accessions, mutant lines and a local check adapted to Al-affected area (Table 1). Seeds of each variety were surface sterilized by soaking in 1% sodium hypochlorite (commercial bleach) for five minutes and were then rinsed five times with sterile water. The seeds were allowed to germinate on moist Whatman® filter paper (Sigma-Aldrich) in sterilized glass Petri dish. Germination took place within 24-36 hours under dark conditions at a temperature of 30^oC.

Determination of the optimum Al concentration for Al-tolerance screening

Five Al concentration levels, 0, 150, 250, 350, 450 and 550 $AlK(SO_4)_2 .12H_2O$ µM, that covered range of Al concentrations used for screening of other cereals, such as sorghum, maize, wheat and rice, were used. A recipe of modified Magnavaca's nutrient solution was obtained from Cornell University (Table 2). The different concentrations of Al solution were freshly prepared from $AlK(SO_4)_2 .12H_2O$ µM and were applied to the basal modified Magnavaca's nutrient solution. The pH of the treatment solutions was adjusted to 4.0 after Al was added, while the pH of the control treatment was adjusted to 5.8 using KOH.

The experiment was laid out in a randomized complete block design (RCBD) with four replications and seven plants per replication. Seven uniformly germinated seedlings were placed in holed Eppendorf tubes supported by acid washed silica sand (0.25mm-2.5mm). The tubes were inserted to neoprene foam to float on the nutrient solution. The nutrient solution was aerated with aquarium pump [Hydrofarm AAPA45L Active Aqua (www.hydrofarm.com)] fitted to infusion tubes clipped to the bottom of 4.5 L plastic tubs by plastic tension clips (Figure 1). The pressure was regulated by the spigot of the air divider attached to the pump and the roller clamp of the infusion set helped to uniformly aerate the hydroponic solution in each tub. The seedlings were exposed to 3600 lux cool white florescent lamp for 16 and 8 hours of

light and dark, respectively. The seedlings were subjected to Al^{3+} treatment containing different concentration levels of Al for 4 days. The pH was continuously monitored and 50% of the hydroponic solution was renewed every other day. The root measurement data was taken after four days.

Screening of tef genotype for Al tolerance

Twenty-eight tef genotypes were evaluated for their Al-tolerance under non-replicated conditions using the same procedures and growth conditions described above but using the optimum Al concentration selected for tef. The root measurement data were collected from seven individual seedlings per each treatment. The accession included were pure lines selected through single panicle selection of the dominant phenotype of the original material received from the gene bank (Ermias et al., 2021).

Data collection and analysis

After four days in the nutrient solution, the seedlings along with the Eppendorf tube were carefully removed from the solution and the primary root and shoot length (mm), the total number and length (mm) of secondary roots, and total number of dead plants from each concentration level was recorded. The mean primary root length was used to compute RTL. The RTL was computed as the ratio of root length in the Al solutions and root length in the control using the following formula:

$$\text{Relative tolerance length (RTL) (\%)} = \left(\frac{Root\ lengrh\ in\ Al\ solution}{Root\ length\ in\ the\ control}\right) x\ 100$$

Analysis of variance and means separation test was performed using the fisher protected Least Significant Difference (LSD) method, at a significant level of α= 0.05 using GenStat Statistical software 19 Edition (GenStat, 2018). In addition, single degree of freedom contrast, and descriptive statistics, was computed. Excel 2013 was used to construct the graphs.

Assessment of haematoxylin staining

In this experiment, the *Eragrostis tef* genotypes: *Holeta Key* (sensitive check) 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^{3+}. The seeds of the four selected genotypes were germinated following the same procedure described above. The germinated seeds were kept in a growth chamber at 25^{o}C for 36 hours in the dark, and seven uniformly germinated seedlings were selected and transferred in nutrient solution supplemented with 0, 150, 250 µm $AlK(SO_4)_2.12H_2O$ µM concentration using holed Eppendorf tubes as described above. The pH of the Al^{3+} solutions and the control were adjusted as indicated above.

After 24 hours, the seedlings were removed from the solution and washed with distilled water for 20 minutes. 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 as described by Cancado et al. (1999). The stain was prepared a day before the actual experiment and was left overnight continuously stirred to dissolve the haematoxylin (Delhaize et al., 1993). The roots were then dipped in the staining solution for 20 minutes and washed with distilled water for 30 minutes with slow agitation to remove the excess stain. The stained roots were kept in a covered glass Petri dish until scoring for colour development was made. The haematoxylin staining pattern of the tip 1.5 cm primary roots were scored under inverted microscope (Nikon), and the degree of staining was scored and images were taken.

Conclusion

In the present study, a highly controlled hydroponic system that allowed efficient discrimination between Al-sensitive and Al-tolerant tef genotypes is adapted. The established optimal Al^{3+} concentration of 150 µM AlK(SO4)2.12H2O for Al-toxicity tolerance screening for tef was verified to adequately discriminated between Al-sensitive and Al-tolerant genotypes on 28 genotypes. 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. Both root growth measurement and haematoxylin staining methods consistently identified Al-sensitive and Al-tolerant genotypes in a set of genotypes evaluated in this study. The use of haematoxylin staining in tef Al-toxicity tolerance screening, however, needs to be preceded by confirmatory test that needs to be carried out on diverse genotypes of tef. Generally, primary objective of this study was achieved, which was to develop a precise phenotyping platform to screen tef genotypes for Al-toxicity tolerance is achieved. Application of this platform for high throughput screening of tef should be used along with digital measurement and image analysis techniques.

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Conflict of Interest

The authors declare no conflict of interest. The institution where the research was done and the funding body are duly acknowledged.

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