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Nutritional quality, response to agronomic biofortification, and mineral bioaccessibility of finger millet genotypes

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I, the undersigned, solemnly declares that this dissertation represents my own work and is not submitted to any other institution elsewhere for the award of any degree, diploma or certificate.

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Table of contents

List of tables **Page**

List of figures **Page**

Abbreviations and acronyms

ANOVA	Analysis of Variance
CC	Central China
CRM	Certified Reference Material
DALYs	Disability-Adjusted Life Years
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
IP6	Inositol hexaphosphate
ISFM	Improved soil fertility management
LOD	Limit of detection
MNDs	Micronutrient deficiencies
masl	Meters above sea level
NE	Northeast
RCBD	Randomized completed block design
SD	Standard deviation
SE	Southeast
SSA	Sub-Saharan Africa
SW	Southwest
TAZ	Total available Zn
TDP	Total daily dietary phytate
TDZ	Total daily dietary Zn
TMAH	Tetra methyl ammonium hydroxide
YS1	Yellow stripe 1

Abstract

Background: High prevalence of zinc (Zn) and iron (Fe) deficiencies which a public health concern in Ethiopia are predominantly a result of cereal based diet with less dense nutrient content. Agronomic biofortification increases micronutrient concentration in the edible part of food crops through the application of mineral fertilizers. But for agronomic biofortification strategy to be effective, targeting food crops and varieties known to adapt in local environment and has a good nutritional quality is important. In addition to the increase in grain mineral concentration in response to agronomic biofortifcation, bioavailability of this mineral to the body system is cruicial.

Objectives: To evaluate the nutritional quality, investigate response to agronomic biofortification and bioaccessibility of different finger millet genotypes of Zn and Fe.

Methods: Fifteen improved genotypes of finger millet were evaluated for their proximate composition, mineral and antinutrient concentration, and mineral bioavailability. Three of these genotypes were then agronomically biofortified with Fe and Zn at two locations (Gojjam and Arsi Negelle) and two slope positions (foot and hill). The genotypes were evaluated for their response towards yield, grain Fe, Zn and antinutrient concentration. Fe and Zn bioaccessibilty was also evaluated.

Result: There was significant variation in protein, fat, fibre, total minerals ranging from 10 to 14.67%, 1.05 to 3.81%, 1.44 to 4.63% and 1.01 to 3.97 %, respectively, as a result of genotypic differences. Similarly, finger millets genotypes had significantly different mineral and antinutritional concentrations ranging from 3762 ±332 to 5893 ±353 mg kg^{-1} for Ca, 19.9 ±1.6 to 26.2 ±2.7 mg kg^{-1} for Zn, 36.3±4.6 to 52.9±9.1mg kg^{-1} for Fe, 36.6 ±11 to 60.9 ±22 µg kg^{-1} for Se, 311.5±2.9 to 341.4±19.9 µg g^{-1} for phytate, 0.16±0.01 to 0.5±0.01 mg g^{-1} for tannin and 1.34±0.2 to 3.39±0.8 mg g^{-1}for oxalate. The combined soil application of Fe and Zn to Meba genotype, Zn to Urji genotype and Fe to Diga-01 genotype increased yield by 51.6, 27.6 and 18.3 %, respectively. Furthermore, grain Zn concentration increased by 18.9 and 20% in response to soil application of combined Fe and Zn and only Zn, respectively. Similarly, 21.4 and 17.8% increase in grain Fe concentration was observed as a result of combined Fe and Zn and only Fe application, respectively. Location but not slope position was a source of variation for both grain Zn and Fe concentrations. Fertilizer treatment showed a significant ($p < 0.001$) variation in Zn bioavailability expressed as a total available Zn (TAZ) ranging from 0.51 to 2.57 mg 300 g^{-1}. Bioaccessible fraction of Zn and Fe increased up to 81 and 88%, respectively, and phytate concentration reduced up to 49% as a result of fermentation. Fermentation of agronomically biofortified finger millet could potentially contributes up to 126% for adult men and 90% for adult women to the total absolute daily Fe requirements, assuming that a person consumes 300g/day in a dry base. Similarly, up to 179% for adult men and 251% for adult women of recommended daily intake of Zn could be fulfilled from agronomically biofortified and fermented finger millet.

Conclusion: Iron and Zn agronomic biofortification could be an effective approach to improve yield, grain mineral concentration as well as their relative bioavailability. It could help to combat Fe and Zn deficiencies through increasing the consumption of more bioavailable Zn and Fe among the society special where finger millet is cultivated as a staple crop. The result suggests that finger millet breeding program should focus on evaluation of nutritional quality alongside agronomic traits. Future studies as well as development programs on agronomic biofortification should consider genotypic and environmental (location and slope position) effects beside the main fertilizer effect, which is a gap in current knowledge base.

Key words: *Agronomic biofortification, bioavailability, consumption, fermentation, iron, zinc*

Chapter 1: General Introduction

1.1 Background and justification

The global population is projected to reach approximately 10 billion in 2050 and, thus, global food and nutrition transition should focuses on unique interventions to feed the dramatically growing population equitably, healthily and sustainably [1]. Regionwise the fastest population growth rate, approximately 114%, is forcasted to be in Sub-Saharan Africa [2, 3]. The World Summit on Food Security, 2009, estimated that at least 70% more food production is required by 2050 to feed the world population by then [2]. It would require an annual increase of approximately 44 million tonnes, which is 38% above current annual increase in food production [4]. However, aspects such as the impacts of climate change, the increased water and land scarcity, urbanization, and use of cereals for fodder and fuel, affect the effectiveness of different effert to respond for future nutritious food demand [5, 6].

On the other hand large population in the globe, particularly in developing countries, is suffering from multiple micronutrient deficiencies (MNDs) [7, 8]. Ethiopia has strong commitment to reduce malnutrition through different strategy including Seqota declaration, national nutrition strategy, food and nutrition policy, etc. However, yet in Ethiopia a high prevalence of Zn (72%) [9] and Fe (34.4%) [10] deficiencies has been reported based on biomarkers of status. Therefore, in such context, improvements in agricultural productivity should lead to greater availability of nutritious food reach in multiple micronutrient at household level. However, soil micronutrient deficiencies are severe in sub-Saharan Africa, where 75% of the total arable land has serious soil fertility problems [11] and deficient in plant bioavailable micronutrients like Zn and Fe [12]. Soil micronutrient deficiencies limit crop productivity and nutritional quality, which together may affect human health [13, 14]. Generally, Zn and Fe deficiencies are most frequently amended by agronomic biofortification through soil application of Zn and Fe fertilizers [15].

Agronomic biofortification is a strategy to increase micronutrient concentration in the edible part of food crops through the application of mineral fertilizers [16, 17]. This approach can enrich food crops with multiple elements at a time and can reach resource poor rural communities, providing they have access to fertilizers. For agronomic biofortification to be effective, targeting food crops and varieties known to well adapt in their local environment and has a good nutritional quality, except for the minerals to be biofortified, is important [18]. Finger millet (*Eleusine coracana* L.), indigenous crop to Ethiopia, is a major staple crop among tribal farming communities in developing countries. Grown in arid regions, they can grow under high temperature, low moisture and poor soils [19], require minimal inputs (fertilizer, herbicides and pesticides), tolerant to disease and possess superior nutritional properties [20, 21]. Finger millet is the sixth most important cereal crop after teff (*Eragrostis tef* Zucc.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), and barley (*Hordeum vulgare* L.) in Ethiopia. It is produced on ~480,000 hectare (ha) of land, yielding about 1.2 million (M) tonnes per annum and ~1.55 M households are directly engaged in its production which increased by 300 % in the previous 20 years [22].

Finger millet also has appreciable nutritional content, e.g. high calcium (Ca) concentration 6600 mg kg^{-1} [23] which is 6-fold greater Ca than in milk 700 to 1100 mg L^{-1} on a volume basis [24]. It is also a good source of other micronutrients [25] and protein (15.6%) [26]. In addition, the grain of finger millet grain is gluten free, which makes it more attractive than wheat to some consumers [27]. However, finger millet is reported to have low Zn: 17.9 to 19.7 mg kg^{-1} and Fe: 32.6 to 38.9 mg kg^{-1} concentrations [28]. Thus, improving finger millets grain Fe and Zn concentration coupled with its other nutritional qualities, could help to combat multiple micronutrient deficiencies (Fe, Zn and Ca).

Previous reports on the impact of Zn and Fe agronomic biofortification, genotype as well as slope position on indigenous crops like finger millet and teff in tropical smallholding farming systems is lacking. However, a report from Ethiopia indicated that wheat yield was more strongly influenced by slope positions than either the nutrient sources or rates [29]. Gashu *et al.* [25] also reported in their subnational scale data from Ethiopia and Malawi that location is a source of variability in cereal grain Zn and Fe concentration. Therefore, site specific fertilizer treatment is strongly recommended.

1.2 Brief literature review

1.2.1 Finger millet

Finger millet (*Eleusine coracana* L.) represents one of the critical plant genetic resources for the agriculture and food security of farmers inhabiting arid, infertile and marginal lands [30]. In the semiarid tropics of Eastern Africa, it is the major staple food for millions of resource poor people and plays an important role in the dietary habits and economy of subsistence farmers [31].

1.2.2 Global cultivation and distribution of finger millet

The oldest domesticated finger millet was found in the archaeological record of a prehistoric site at Axum, Ethiopia, dating back some 5000 years and it resembles race plana (a highly evolved race) which is the principal finger millet still grown in Ethiopia [32].

Finger millet cultivation extends in Africa from Nigeria eastwards to Eritrea and south towards South-West Africa and Natal in South Africa [33]. It was introduced to India at a very early date, probably over 3000 years ago [32, 34] and now it is an important staple food in some places, particularly in the hilly country in the north and the south of India.

Finger millet is widely cultivated in the tropical and sub-tropical regions of Africa and India [35]. It is largely produced in India, Uganda, Tanzania, Kenya, Ethiopia, Rwanda, Zaire, Zambia, Zimbabwe, Eritrea, Somalia, China, and Myanmar [31]. The precise global area under finger millet and its production is not known because this crop has often been grouped and reported with other millets such as Proso millet (*Panicum miliaceum*), pearl millet (*Pennisetum glaucum*), Foxtail millet (*Setaria italica*), Barnyard (Sawa) millet (*Echinochloa colona*), Kodo millet (*Paspalum scrobiculatum*). An estimated total production of millet is 33,634,825 tons worldwide [36]. It is estimated that the share of the global finger millet production is about 12.8% of the millet, thus, the finger millet share is estimated to be 4,305,258 tons [37].

1.2.3 National finger millet production

In Ethiopia, finger millet is the sixth important crops after teff (*Eragrostis tef* Zucc.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), and barley (*Hordeum vulgare* L.). It was produced on 480,343 ha of land, from which 1.2 M tons were obtained at the national level per year, equivalent to 30% of global production [22]. Nationwide 1.5 M households are directly engaged in finger millet production and the production growth has been increasing by 300 % in the previous 20 years [22].

Although the yield potential reported is more than 6 tons/ha [38], the current national average is only limited to 2.95 tons ha^{-1} [22]. This could be mainly due to the lack of widely adaptable improved varieties, limited management recommendations, farmer's perceptions toward the crop, lack of financial services etc [38, 39]. Moreover, breeding efforts in finger millet has been limited, in general, and majority of farmers are generally growing unimproved and low yielding cultivars [40, 41]. This necessitates development of stable high yielding cultivars with desirable nutritional traits.

1.2.4 Finger millet's agronomic merits

Finger millets are small seeded plants, are drought tolerant crops found in tropical and sub-tropical regions of the world and grow very well in harsh environments where other crops usually fail [42]. They represents one of the critical plant genetic resources for the agriculture and food security of populations inhabiting arid, infertile and marginal lands [43, 44]. Due to their ability to withstand drought, coupled with the production of substantial yields in a wide range of soils and climates, millets have become a staple food in many African and Asian countries [45] where they are mostly cultivated. Generally, the major attributes of finger millet are its adaptability to adverse agro-ecological conditions with minimal inputs (fertilizer, pesticide, herbicides etc), tolerance to moisture stress, productivity on marginal land where other crops cannot grow, disease tolerance and tolerance to acidic soil [46]. It is, also, cab be easily stored for a longer period than most cereals and therefore it is considered as a useful famine crop [47].

1.2.5 Finger millet's nutritional merits

The richness in calcium, polyphenol, and protein content in finger millet make it unique among the cereals [48, 49]. Finger millet calcium concentration is about 400% higher as compared to other millet families (Pearl, Proso, Foxtail, and Kodo millets) and about 90 to 230% higher as compared to other cereals like wheat, barley, sorghum, maize, rice, and rye [50], and about 5 folds of calcium in milk (700 to 1100 mg L^{-1}) [24]. It is also a good source of other micronutrients [25] and protein (15.6%) [26]. In addition, the grain of finger millet grain is gluten free, which makes it more attractive than wheat to some consumers [27]. Finger millet contains 44.7% compared to 33.9% of essential amino acids that is found in the Food and Agricultural Organization's reference protein as a proportion of the total amino acids content [51, 52]. Finger millet has lipid content of 1.5 to 5.3% and the main fatty acids found in millets are palmitic, oleic and linoleic acid [53].

1.2.6 Effectiveness of Agronomic Biofortification Strategy in Fighting against Hidden Hunger

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Abstract

Micronutrient deficiencies (MNDs), also known as hidden hunger, affect more than a quarter of the global population. Agronomic biofortification helps to increase the concentration of a target mineral in food crops and improve human mineral dietary intake. It is a means of providing nutrient-dense foods to a larger population, especially among rural resource-poor settings, providing that they have access to mineral fertilizers. However, the feasibility of agronomic biofortification in combating hidden hunger depends on several factors in addition to fertilizer access, including crop type, genotype, climate, soils, and soil mineral interactions. Consideration of its effectiveness in increasing human mineral intake to the daily requirements and the improvement of human health and the cost-effectiveness of the program is also important. In this paper, we review the available literature regarding the potential effectiveness and challenges of agronomic biofortification to improve crop micronutrient concentrations and reduce hidden hunger.

Keywords: agronomic biofortification; dietary intake; effectiveness; fertilizers; micronutrient deficiencies

1.2.6.1 Introduction

Micronutrient deficiencies (MNDs), also known as ‘hidden hunger’, occur when dietary intakes of vitamins and mineral micronutrients are not adequate for optimal human health. MNDs are a public health concern worldwide and have been the focus of intensive research for many years. It is estimated that more than a quarter of the global population is affected by the deficiency of one or more micronutrients [1]. MNDs are a risk factor for many diseases, contributing to the existing high rates of morbidity and mortality. For example, MNDs can lead to reduced resistance to infections, which can cause severe illnesses and developmental challenges, including anemia, mental retardation, blindness, and spinal and brain birth defects. The most prevalent forms of MNDs are iron (Fe), iodine (I), zinc (Zn), and vitamin A [2,3]. In terms of the loss of healthy life years, the deficiency of these micronutrients is responsible for 1.5–12% of the total disability-adjusted life years (DALYs) lost in sub-Saharan Africa (SSA) [4]. It has been estimated that under-nutrition and MNDs, combined, cost the world up to USD 3.5 trillion every year [5]. The research also shows that MNDs among women of reproductive age lead to undesirable birth outcomes in new-borns, together with a higher risk of physical and cognitive impairment, leading to economic stagnation and intergenerational poverty [6].

Understanding the etiology of MNDs is vital in the process of designing and implementing strategies for the prevention of diet-related diseases [7]. MNDs can be addressed through the implementation of programs. Dietary diversification, food fortification, supplementation, and the genetic and agronomic biofortification of food crops are among the strategies. In addition to improving micronutrient intake, dietary diversification has the potential to improve the intake of many food constituents at the same time. It is typically considered to be the most sustainable and preferred strategy compared to the others. However, the availability and affordability of diversified foods are often barriers in resource-poor societies. Changes in dietary patterns through nutrition education and behavioural change communication also make the strategy tough to achieve [2].

Supplementation of high dose vitamins and minerals is a strategy that can quickly improve the micronutrient status of individuals or a targeted population [2]. However, supplementation depends on the availability of supplements to the individual at the correct level. In addition, it is not necessarily sustainable because it does not address the root cause of the particular MND or multiple MNDs. Nutrients from supplements can also show different physiological responses and absorption rates than nutrients in food [2]. The procurement of micronutrients in a relatively expensive pre-packaged form is also a challenge in resource-poor communities [2].

Food fortification can have a wider impact and is potentially more sustainable than supplementation. However, fortification is dependent on centrally processed food vehicles and requires the engagement of food-processing industries. Furthermore, some communities can be difficult to reach through the implementation of food fortification, especially those that consume locally produced food sources. The sustainability of the mineral supply to food industries, the bioavailability of fortified minerals, and possible sensory changes as a result of fortification could be additional challenges to this strategy [2]. Overall, food fortification, supplementation, and diet diversification strategies may work well only in urban settings [8,9].

Improvement in the quantity as well as the quality of essential nutrients in the edible portions of crops during plant growth either genetically and/or agronomically is known as biofortification [10]. Biofortification that is achieved through genetic engineering or classical breeding is called genetic biofortification, while agronomic biofortification involves the application of a micronutrient fertilizer either to the soil (basal application) or application directly to the leaves of the crop (foliar application) [11,12]. The focus of this review is agronomic biofortification.

1.2.6.2 Agronomic Biofortification

Agronomic biofortification is the strategy of increasing the micronutrient contents in the edible parts of food crops through the basal and/or foliar application of mineral fertilizers [11,12]. Agronomic biofortification can enrich crops with multiple elements, but the most common ones are Fe, Se, Zn, and I. It may be a suitable approach to reach resource-poor rural populations, provided they have access to chemical fertilizers. Soil-to-plant transfer and the accumulation of minerals in the edible portion of food crops determine the success of biofortification. In addition, the bioavailability of minerals from biofortified crops in the body influences the effectiveness of biofortification programs.

1.2.6.3 Evidence from Agronomic Biofortification

Agronomic biofortification has mainly been carried out on staple cereal crops like rice, wheat, and maize because they dominate diets worldwide, especially among groups vulnerable to MND. Dimpka and Bindraban [13] recommend that micronutrient fertilization should improve the yields as well as the nutrient contents of crops. This is because fertilization programs in developing countries typically focus on nitrogen, phosphorus, and potassium (NPK) and/or sulfur (S) fertilizers, yet crop yields can still be limited by multiple soil micronutrient deficiencies [14]. Basal application of multiple elements in small amounts to the soil has, therefore, been recommended as a sustainable strategy to increase both the yields and the nutrient quality of crops [14–16].

Most research on agronomic biofortification has focused on Se and Zn, and these micronutrients are the focus of this review. Selenium is an essential trace element with many roles in human health; however, it has no known biological roles in plants. Blending or granulating Se with macronutrient fertilizers can be highly effective [12]. For example, crops in Finland showed a 15-fold increase in their Se concentration due to the application of Se with NPK fertilizers [17]. Similarly, in a recent study from Malawi, an 88–97% increase in the Se concentration of maize grain was observed due to the application of 20 g ha^{-1} Se fertilizer [18]. Grain Se increased by about 10-fold as a result of 25 g ha^{-1} Se fertilizer application in Brazil [19]. De Lima Lessa et al. [20] and Chilimba et al. [18] showed approximately linear increments of grain Se concentration with increased Se fertilizer application in their studies conducted in Brazil and Malawi, respectively. Other studies from Kenya and Australia also reported linear increases in grain Se concentrations with increases in the Se fertilizer application dose [21]. On the other hand, studies that compared the effects of Se chemical forms (nanoparticle, sodium selenite, and sodium selenite) on faba bean seed [22] and tomato fruit [23] Se concentrations reported that nanoparticles exerted the smallest effects compared to the other chemical forms. In general, multiple previous studies have reported the positive impact of Se agronomic biofortification on grain Se concentration (Table 1.1). However, there was no evidence that Se fertilizer application had an effect on crop yield in these studies.

Table 1.1. Previous reports on impact of Se agronomic biofortification on grain Se concentration.

No.	Crop	Application Method	Application Rate	Grain Se Increase (%)	Reference
1	Wheat	Basal	55.4–21.6 mg ha^{-1} elemental Se	283–1650	[24]
2	Rice	Foliar	30 g ha^{-1} Na_2SeO_3	259	[25]
3	Wheat	Basal	5 g ha^{-1} elemental Se	137	[26]
		Foliar	5 g ha^{-1} elemental Se	51–155	
		Basal and foliar	A total of 10 g ha^{-1} elemental Se	61–364	
4	Soybean	Basal	80 g ha^{-1} Na_2SeO_4	290–331	[27]
5	Maize	Basal	5–20 g ha^{-1} Na_2SeO_4	25–227	[28]
		Foliar	5–20 g ha^{-1} Na_2SeO_4	423–819	
6	Faba bean	Foliar	1 L m^{-2} Se nanoparticles (90 nm) (concentration = 100 mg L^{-1})	1360	[22]
			1 L m^{-2} sodium selenite (concentration = 220 mg L^{-1})	3799	
			1 L m^{-2} sodium selenate (concentration = 240 mg L^{-1})	7426	

In contrast to Se, Zn is an essential plant nutrient and a yield-limiting factor in many production systems. Cakmak [12] showed that Zn fertilization enhances yield as well as crop Zn concentrations. Previous studies reported the positive impact of Zn agronomic biofortification on both yield and grain Zn concentration (Table 1.2). Joy et al. [29] systematically reviewed studies and reported an incremental effect of Zn fertilizer application on Zn concentrations in maize (20%), rice (7%), and wheat (19%) in 10 African countries. The same review indicated that foliar Zn application resulted in even higher grain Zn concentrations in maize (30%), rice (25%), and wheat (63%). Moreover, the chemical form of Zn has been reported to have a significant impact on both crop yield and grain Zn concentration. For instance, Umar et al. [30] reported that the application of Zn nanoparticles on maize was more effective in improving both the grain yield and Zn concentration. Similar studies on rice [31] and wheat [32] have reported that Zn nanoparticles were effective at increasing grain Zn concentration, but the yield remained unaffected (Table 1.2).

Table 1.2. Previous reports on impact of Zn agronomic biofortification on crop yield as well as grain Zn concentration.

No.	Crop	Application Method	Application Rate	Yield Increase (%)	Grain Zn Increase (%)	Reference
1	Maize	Basal	30 kg ha^{-1} elemental Zn	11	15	[33]
2	Chickpea	Basal	25 kg ha^{-1} $ZnSO_47H_2O$	10.2	24.9	[34]
		Foliar	0.5% (*w/v*) $ZnSO_47H_2O$	9.2	35.4	
		Basal and foliar	25 kg ha^{-1} and 0.5% (*w/v*) $ZnSO_47H_2O$	14.3	39.1	
3	Rice	Foliar	0.5% (*w/v*) $ZnSO_4.7H_2O$	10	66	[35]
4	Rice	Basal	20 mg elemental Zn per 1 kg soil	23.5	80.4	[36]
5	Wheat	Basal	25 kg ha^{-1} $ZnSO_47H_2O$	5	18	[37]
		Foliar	0.5% (*w/v*) $ZnSO_47H_2O$	3	47	
6	Rice	Basal	5 kg ha^{-1} elemental Zn		26.5	[38]
		Foliar	0.5% (*w/v*) $ZnSO_4.7H_2O$		79.5	
		Basal and foliar	5 kg ha^{-1} elemental Zn0.5% (*w/v*) & $ZnSO_4.7H_2O$		89.8	
7	Maize	Basal	ZnO nanoparticle (105 nm) (8 kg Zn ha^{-1})	44	59	[30]
			ZnO (8 kg Zn ha^{-1})	11	28	
		Foliar	ZnO nanoparticle (105 nm) (2% solution)	33	82	
			ZnO (2% solution)	11	38	
8	Rice	Basal	25–100 mg Zn nanoparticle (30 ± 10 nm) kg^{-1} soil	88.3	24.2	[31]
			25–100 mg Zn from $ZnSO_4.7H_2O$ kg^{-1} soil	86.5	12.6	
9	Wheat	Basal	10–1000 mg ZnO nanoparticle (<100 nm) kg^{-1} soil	5.6–56	23.5–230	[32]
			10–1000 mg Zn from $ZnSO_4.7H_2O$ kg^{-1} soil	8.8–55	12.6–142	

Overwhelming evidence from many countries has shown that the application of Zn fertilizer on Zn-deficient soils improves the yield and/or grain Zn concentration [11,39–52]. However, one study in Pakistan reported little or no significant effect of Zn fertilizer application on rice yield or grain Zn concentration [35]. This was due to the presence of high DTPA-extractable Zn (2.2 to 6.5 mg kg^{-1}) in the soil, while the level of DTPA-extractable Zn in soil considered to be critical for Zn deficiency in rice is 0.5–0.8 mg Zn kg^{-1} [53]. Zia et al. [54] also reported no significant effect on wheat grain Zn concentration as a result of soil Zn application, which, again, may be linked to soil properties.

There are fewer studies on the effect of Fe agronomic biofortification compared to Se and Zn. For example, a study from India reported a 13% yield and 2-fold wheat grain Fe concentration increase due to Fe fertilization [37]. Similarly, another study on finger millet reported a positive impact of Fe fertilization on both grain yield and Fe concentration (Table 1.3). In contrast, Zhang et al. [55] and Pahlavan-Rad and Pessarakli [56] from China and Iran observed 36% and 21% wheat grain Fe concentration increases, respectively, but the yield remained unaffected. However, studies from Turkey and Canada on the Fe biofortification of barley and wheat, respectively, showed neither yield nor grain Fe concentration improvement [39,57]. This was due to two reasons. First, graminaceous species release phytosiderophores (Fe-mobilizing compounds) to solubilize and absorb Fe from soils with low Fe concentrations, and thus, they can maintain adequate plant growth by satisfying Fe demand without the requirement of Fe fertilization [54,58,56]. The second reason is that when applied to calcareous soils, Fe is rapidly converted into unavailable forms, and the poor mobility of Fe in phloem makes Fe fertilization unsuccessful [11,59]. Furthermore, the crop response to Fe fertilization is more dependent on the synergetic effect of nitrogen fertilizer [39,60]; the details are presented in section 1.1.6.5. *B*. The chemical form of Fe is also reported to have a significant impact on both the crop yield and grain Fe concentration. For instance, foliar application of Fe nanoparticles showed a significantly higher impact on wheat grain Fe concentration, but not yield, compared to Fe-EDTA and $FeSO_4$ [61]. On the other hand, Dhaliwal et al. [62] and Taskin and Gunes [63] reported significantly higher yields, but not grain Fe concentration, in chickpea and wheat, respectively, as a result of foliar application of Fe nanoparticles compared to $FeSO_4$ application (Table 1.3).

Table 1.3. Previous reports on impact of Fe agronomic biofortification on crop yield as well as grain Fe concentration.

No.	Crop	Application Method	Application Rate	Yield Increase (%)	Grain Fe Increase (%)	Reference
1	Wheat	Foliar	50 mg Fe L^{-1} from 1 to 3 sprays		1.3–22	[64]
2	Wheat	Foliar	6 g L^{-1} (0.84 kg ha^{-1}) FeO_3 nanoparticle	11.4	17	[61]
			12 g L^{-1} (1.1 kg ha^{-1}) elemental Fe from $FeSO_47H_2O$	13	11.3	
			12 g L^{-1} (1.1 kg ha^{-1}) elemental Fe from Fe-EDTA	3.8	5.1	
3	Finger millet	Basal	4 kg ha^{-1} elemental Fe from $FeSO_47H_2O$	18.3	17.8	[65,66]
4	Chickpea	Foliar	0.5% $FeSO_47H_2O$	7.1	−2.8	[62]
			0.5% FeO_3 nanoparticle	43	0.16	
5	Wheat	Foliar	0.2% Fe from $FeSO_47H_2O$	6.6	13.2	[63]
			0.2% nano zero-valent Fe (29 to 50 nm)	−1.9	12.6	

1.2.6.4 Effect on Human Nutrition and Health

It is suggested that agronomic biofortification potentially improves the daily intake of minerals and helps to alleviate MNDs [18,67]. However, the effectiveness of agronomic biofortification on the improvement of human micronutrient status and health is currently less well studied. The only large-scale effectiveness study that linked agronomic biofortification to the improvement of human Se status and health was reported from Finland. The average dietary intake of Se was 0.04 mg Se/day/10 MJ when Finland started the agronomic biofortification of Se in 1985. After six years of extensive application, the average dietary intake of Se was enhanced to 0.12 mg Se/day. After four years, the mean human plasma Se concentration increased from 0.89 µmol/L to 1.50 µmol/L. The authors concluded that the nationwide agronomic biofortification of Se was found to be effective and safe for increasing the Se intake of the whole population [17]. A randomized control feeding trial study in Malawi to test the effectiveness of the consumption of Se-biofortified maize showed significant increases in serum Se concentrations over a two-month intervention period from 57.6 (17.0) µg L^{-1} (n = 88) to 107.9 (16.4) µg L^{-1} (n = 88) among WRA and from 46.4 (14.8) µg L^{-1} (n = 86) to 97.1 (16.0) µg L^{-1} (n = 88) among SAC without a significant increase among their counterparts who received non-biofortified maize [68].

Lowe et al. [69] also reported an additional daily Zn intake between 3 and 6 mg for refined and whole grain flour, respectively, as a result of an average flour consumption of 224 g d^{-1} of Zn biofortified wheat flour. After 4 weeks of consumption, a significant increase in the plasma Zn concentration of 41.5 μg L^{-1} was observed. A study investigated the impact of Zn-biofortified wheat flour consumption on the Zn status of Pakistani adolescent girls (n = 517) and indicated a moderate increase in the intakes of Zn (1.5 mg/day) and Fe (1.2 mg/day) but did not have a significant effect on plasma Zn concentrations [70]. A study on the efficacy of Fe-biofortified pearl millet in improving attention and memory in Indian adolescents (n = 140) indicated a 30% hemoglobin increase due to four months of consumption of Fe-biofortified pearl millet (Fe = 86 ppm) compared to a non-biofortified version (Fe = 21–52 ppm) [71].

Ex ante analysis of the potential of Zn fertilizers to alleviate human dietary Zn deficiency, focusing on ten African countries where dietary Zn supply is low, showed considerable reductions in the DALYs lost due to Zn deficiency, with 0.5–18.6% in Burkina Faso, 8.8–53.8% in Ethiopia, 1.2–22.8% in Ghana, 2.9–28.9% in Kenya, 9.5–29.4% in Malawi, up to 22.2% in Mali, 2.2–24.4% in Nigeria, 2.1–32.7% in Senegal, 1.8–25.8% in Tanzania, and 6.6–27.7% in Zambia. The cost per DALY saved ranged from USD 624 to 5,893 and from USD 46 to 347 due to granular and foliar fertilizer applications, respectively. The scenario of foliar Zn application is predicted to be cost-effective in all nations according to the WHO standard [29]. Joy et al. [**72**] also reported that the application of Zn fertilizers to wheat in the Punjab and Sindh areas of Pakistan could increase the dietary Zn supply from ~12.6 to 14.6 mg $capita^{-1}$ d^{-1}, with a cost per DALY saved of USD 461–619. Another ex ante analysis aiming to quantify the potential cost-effectiveness of the agronomic biofortification of staple crops with Zn for alleviating Zn deficiency in Ethiopia indicated that biofortification with granular Zn could reduce the burden of Zn deficiency by 29 and 38% with a cost of USD 502 and USD 505 to avert each DALY lost under pessimistic and optimistic scenarios, respectively. Foliar Zn application was predicted to cost USD 226 and USD 496 to avert each DALY lost under pessimistic and optimistic scenarios, respectively [73].

Another study that explored the potential of the agronomic biofortification of rice with Zn and Fe to alleviate human dietary Zn and Fe deficiency was conducted in four regions of China: Northeast (NE), Central China (CC), Southeast (SE), and Southwest (SW). The results showed considerable (0.92%–28%) reductions in the DALYs lost due to Fe deficiency. Similarly, reductions in the DALYs lost due to Zn deficiency were in the range of 3%–55%. The cost per DALY saved ranged from USD 376 to 4,989, from USD 194 to 2,730, and from USD 37.6 to

530 for single, dual, and triple foliar Fe and Zn applications, respectively. The combined foliar spray of Fe and Zn in CC, SE, and SW was found to be cost-effective according to The World Bank standard [74].

1.2.6.5 Potential Challenges to Agronomic Biofortification

A. Mineral Fertilizer Manufacturing

One of the major challenges of agronomic biofortification as a strategy is the manufacturing of fertilizers containing a suitable quantity of mineral micronutrients, especially in many developing countries, where most fertilizer is imported. Strategies aiming to reduce MNDs are likely to be more effective where the intervention is case-sensitive in local situations [21,75]. To produce a fertilizer blend for a specific location is likely to require the close involvement of public and private fertilizer production and distribution sectors.

B. Mineral Fertilizer Application Method

There are two approaches for the application of mineral fertilizers - foliar and basal application. The two approaches have their costs and benefits in terms of logistics, economic feasibility, and final grain mineral concentration.

In the short term, foliar Zn applications are more effective than soil applications at increasing grain Zn concentrations in wheat [35,54]. For example, foliar Zn application to rice and wheat represents an effective agronomic practice to enhance the grain Zn concentration up to 66%, while soil application has no effect [35,41]. Soil applications of Zn are less effective than foliar applications to increase grain Zn concentration. The study by Joy et al. [29] indicated that soil Zn application led to increases in the median Zn concentrations in maize, rice, and wheat grains of 23%, 7%, and 19%, respectively, while foliar application led to increases of 30%, 25%, and 63%, respectively. The authors suggested that Zn fixation in the soil makes foliar applications more cost-effective than soil applications; however, the deployment might be more complicated. Botoman et al. [33] reported that many studies on soil Zn applications are underpowered to detect small increases in crop Zn concentration; they reported a 15% increase in maize Zn concentration as a result of 30 kg ha^{-1} elemental Zn application. A study from Zimbabwe aimed at quantifying the potential health benefits of alleviating dietary Zn deficiency with soil-applied Zn fertilizer and improved soil fertility management (ISFM) to increase maize grain Zn concentration reported that soil Zn fertilizers were estimated to increase the dietary Zn supply from 9.3 to 11.9 mg Zn $capita^{-1}$ day^{-1}, reduce the dietary Zn deficiency prevalence from 68% to 31%, and save 6576 DALYs lost per year. On the other hand, soil Zn fertilizer, together with ISFM, is estimated to increase the dietary Zn supply from

9.3 to 12.5 mg Zn capita^{-1}day^{-1}, reduce the dietary Zn deficiency prevalence from 68 to 25%, and save 7606 DALYs lost per year [76]. Therefore, the report indicates strong effects of other ISFM approaches on the effectiveness of soil-applied Zn.

One benefit of soil application of Zn fertilizer is its potential residual effects in subsequent cropping seasons. For example, Narwal et al. [37] reported that soil application of Zn to wheat has a significant effect for multiple years and could be more effective and economical for wheat in the long run as compared to foliar application. Another study reported that soil application of 28 kg ha^{-1} $ZnSO_4$ fertilizer was an effective strategy to correct soil Zn deficiencies for about 7 years [**77**]. Similarly, Frye et al. [78] reported the residual effect ranging from 4 to 5 years as a result of soil application of 34 kg ha^{-1} $ZnSO_4$ fertilizer. Similar researchers reported that soil application of $ZnSO_4$ ranging from 18 to 28 kg ha^{-1} is adequate to correct Zn deficiency in plants for four to seven years [79–81]. Therefore, the argument is, if the application of Zn fertilization is planned for more than one season, basal application could be a more cost-effective method due to its residual effect, whereas foliar application may provide the highest grain Zn concentration for a single production season.

Some studies have indicated that the combined application of soil and foliar Zn and Fe are more effective than a single soil or foliar application. The results indicate an increase from 25 to 100% grain mineral content due to combined soil and foliar fertilization application [35,38,41,45,53,82]. However, it is very crucial to consider the soil type effect since the combined foliar and basal application method of Zn on wheat is reported to highly depend on the soil type [54].

Ngigi et al. [28] suggested that foliar application of Se was more effective than soil application for maize and beans. However, it is important to consider that Se can act both as an antioxidant and a prooxidant, and in its concentrated form, Se is toxic [83], therefore, blended or granular Se applied to soils is the only safe approach for farmers. Ros et al. [84] argued that soil application of Se could result in similar responses to foliar-applied Se fertilizer, and the effects of soil-applied Se lasted longer than foliar-applied Se since residual effects were observed for up to 4 years. Chilimba et al. [18] also reported no significant difference between basal and foliar application of Se. They reported for each gram of Se ha^{-1} applied, the Se concentration in maize grain increased by 11–29 µg Se kg^{-1} and by 11–33 µg Se kg^{-1} for foliar and basal applications, respectively. The only comprehensive nationwide experience that has deployed

Se fertilization with basal application, in Finland, reported a 15-fold increase in crop Se content [17].

Soil application of Fe usually has no or only limited residual effects, as Fe^{2+} is rapidly converted into Fe^{3+} in soils; therefore, foliar application has been considered the most effective method, especially for plants that develop grain months after germination [35,37,56,59]. However, other studies found that neither soil nor foliar application of Fe fertilization was an effective method to enhance wheat, barley, or oat Fe concentrations [39,57]. In contrast, regular foliar Fe application could result in a potential environmental hazard [85]. Manzeke-Kangara et al. [60] and Aciksoz et al. [39] argued that the efficiency of soil Fe application is more dependent on other factors, especially the integration of N fertilization and ISFM, compared to the Fe fertilizer application method (foliar or basal).

Studies have suggested the potential of a multi-mineral agronomic biofortification strategy to address multiple mineral deficiencies, based on a site-specific biofortification strategy. Mao et al. [75] reported that combined Se, Zn, and I fertilizers were as effective as singly-applied fertilizers when applied to maize, soybean, potato, and cabbage. This suggests that multi-mineral agronomic biofortification has the potential to address multiple MNDs simultaneously. However, knowledge about the elemental antagonistic and synergetic interaction effect is very critical. Pahlavan-Rad and Pessarakli, [56] reported 8% and 13% increases in wheat grain Fe and Zn concentrations, respectively, as a result of Fe and Zn interaction in their study on the combined application of Fe and Zn fertilization. Even though the mechanism of Zn and Fe interaction is not well understood [86], it has been reported that Zn treatment resulted in Fe accumulation in soybean roots and increased root-to-fruit Fe translocation in tomato plants [87].

C. Mineral Interaction Effect

Interactions between phosphorus (P) and Zn and between P and Fe in soils and plants have long been recognized and well documented. Studies have reported that high soil P levels can negatively affect Zn and Fe uptake by crops by inhibiting the mycorrhizal colonization of roots and resulting in impaired nutrient uptake [88,89]. Multiple studies have reported that P deficiency in soil results in a higher accumulation of Zn, whereas Zn deficiency in soil leads to a higher accumulation of P in plants [90–92]. Similarly, Fe deficiency stimulates the absorption of P in both roots and shoots [93–96]. Erdal [97] reported that soil Zn application enhances wheat grain Zn, and at the same time, significantly reduces grain P concentration. Another study also reported the association between Zn fertilization and a reduction in the phytic acid in rice grain, ranging from 14.8 to 30.4% [38]. These findings suggest that agronomic biofortification with Fe and Zn might also be a useful strategy to reduce antinutritional factors, such as phytate, in addition to increasing the grain mineral concentration.

A study that employed a factorial design involving the application of N up to 60 kg ha^{-1} and Zn up to 10 kg ha^{-1} on pearl millet indicated that the highest grain Zn concentration was observed at the application of 20 kg N ha^{-1} and 5 kg Zn ha^{-1} [98]. Similarly, the Zn uptake rate was enhanced by 4-fold due to the increased N application [99]. Similarly, multiple studies have indicated that N significantly enhances grain Zn [34,100] and Fe [36,39,60,101] concentrations. Nitrogen can increase the activity of transporter proteins and nitrogenous compounds, like nicotianamine, which helps to maintain Zn root uptake and shoot translocation [101,102], and by increasing the activity and abundance of Fe transporter proteins, such as yellow stripe 1 (YS1), in root cell membranes [103,104], which positively affects the root uptake and shoot transport of Fe. Similarly, the Se concentration of rice grains increased by 54.6% as a result of a combined Se and N application compared to only Se application as a fertilizer [19]. These findings suggest the application of Zn, Fe, and Se as a fertilizer is more effective when they are applied along with N fertilization and ISFM.

D. Environmental Impact

Uncontrolled and excessive mineral fertilizer use could cause contamination risk in the environment from the minerals of interest. It has been reported that about 28 tons of extra Cu per year is released into the soil in parts of the United Kingdom as a result of Cu fertilizer [105]. Furthermore, the long-term application of mineral fertilizer was reported to adversely affect important rhizospheric microorganisms that play major roles in plant nutrition and health [106–108]. In such cases, it is recommended to use nanoparticle fertilization, which potentially reduces the release of excessive mineral fertilizers into the environment. For instance, the application of Fe oxide nanoparticles on wheat [109], Zn oxide on maize [30], and Se nanoparticles on soybean [110] effectively improved grain Fe, Zn, and Se concentrations, respectively, without extra mineral release into the environment.

1.2.6.6 Mineral Fertilizer Application Timing

The timing of mineral application is always critical for its effectiveness in improving grain mineral concentration and/or yield. Foliar Zn applications resulted in a marginal effect on rice grain Zn when applied at the stem elongation plus the booting stage, but much greater increases in grain Zn concentration were achieved when foliar Zn application was performed when the crop had reached the milk stage [35]. Fang et al. [111] suggested foliar Zn application at the heading stage as the best practice to improve the Zn concentration of white rice. Sharma et al. [112] and Zeidan et al. [113] argued that the application of Zn fertilization on wheat at the grain-filling stage is an ideal method to increase grain Zn concentrations. The application of Zn fertilizer at the flowering and pod formation stages of chickpea were reported to result in the maximum grain Zn concentration [34].

The application of Se fertilizer during the vegetative stage of crops has been observed to enable and stimulate the quick uptake of Se by the crop [83], although the optimal timing will likely be context-specific. Wheat grain Se concentration increased more when Se fertilizer was applied at the booting stage compared to the earlier jointing stage [114]. Deng et al. [115] also reported that Se fertilizer treatment on rice resulted in a 2-fold higher grain Se concentration at full-heading application compared to late-tillering application. The application of Se fertilizer at flowering increased grain Se concentrations more than when Se was applied at earlier stages in winter wheat [116]. Galinha et al. [117] reported that Se fertilizer application at the booting stage was more effective in enhancing wheat grain Se concentration compared to the grain-filling stage.

The maximum Fe concentration was achieved from foliar application during the maximum tillering stage [118]. The combination of soil Zn application at sowing and foliar application of Zn along with urea at the flowering and pod formation stages can be the best strategy to enhance Zn and Fe contents in chickpea grain [34]. A study showed that the grain-filling stage of wheat might be the best crop development stage to apply Fe fertilization to attain the maximum grain Zn concentrations [113]. This finding suggests that it is very critical to understand crops as well as the genotype timing of mineral mobilization, remobilization, and translocation within the plant to achieve the best results with respect to grain mineral concentration.

1.2.6.7 Cost of Mineral Fertilizer

Farmers might be willing to pay for the extra cost incurred due to biofortification for minerals that can increase yields, like Zn. However, covering the cost of minerals that do not increase yield, such as Se, is a challenge for fertilizer policy discussions. Given that Se deficiency leads to health complications, it may be appropriate for public health policies to consider whether agronomic biofortification is cost-effective. Further, Joy et al. [68] argued that the application of 7.3 kilo tons of $ZnSO_4H_2O$ on wheat per year increased the yield by ~7.5% and dietary Zn by 15.9% $capita^{-1}day^{-1}$ and reduced the prevalence of Zn deficiency by ~50%. Therefore, consideration of the cost-effectiveness of minerals like Zn and Fe should not be seen only from the perspective of their impact on the crop yield, but should also include the cost per DALY saved. Manzeke-Kangara et al. [76] argued that the cost of Zn fertilization in Zimbabwe for maize was not likely to be as useful as investing in nitrogen, due to the yield gaps.

1.2.6.8 Conclusions

A large number of studies have investigated the impact of agronomic biofortification with Se, Fe, and Zn on grain mineral concentration, primarily on staple cereal crops. Most studies have suggested that agronomic biofortification is likely to be a feasible strategy to enhance grain mineral concentrations, especially among rural resource-poor settings, providing that they have access to mineral fertilization. It is also clear that agronomic biofortification is dependent on many factors, like the timing and method of mineral application, mineral–mineral and mineral–soil interactions, and the adoption of ISFM and other practices. It is, therefore, important to have the right information on these factors prior to the intervention in order to make agronomic biofortification successful. Very few studies have tried to investigate the effectiveness of agronomic biofortification on the improvement of human dietary intake and health, and further studies are required. Reports on the effectiveness of agronomic biofortification on indigenous crops, like finger millet, teff, and amaranth, in tropical smallholding farming systems are lacking. However, these crops are highly adaptive to the local climate and efficiently withstand biotic and abiotic stresses, which is crucial in the effectiveness of agronomic biofortification. In general terms, it is possible to conclude that agronomic biofortification can be a supplementary strategy to combat MND among resource-poor rural settings where people are dependent on their own produce as a food source, and in which other interventions, like supplementation and food fortification, may not be suitable.

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1.2.6.9 References

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1.3 Statement of the problem

Compared to more common cereals like wheat, rice and maize, little is known about the varietal differences in nutritional composition, mineral bioaccessibility and response to Zn and Fe agronomic biofortificaation of finger millet genotypes in Ethiopia. This, along with public perception and technological limitation in processing finger millet, have for long restricted its more wide spread consumption in Ethiopia, despite its nutritional qualities. Limited information availability on the nutritional benefit of finger millet could be the reason for lack of interest in finger millet. Although Ethiopia is the centre of origin and domestication for finger millet, comprehensive studies on finger millet diversity are generally limited [39],

In Ethiopia, finger millet occupies diverse agro-ecologies with a vast range of genetic variability and is intensively cultivated in mid and lower altitude regions of Tigray, Gojam, Gonder and Wollega where it constitutes up-to 30 % of the total cereal production [22]. The Ethiopian Central Statistical Authority data reveals that finger millet production is increased by ~300 % in the previous 20 years and the area devoted to finger millet production as well as its productivity is increasing [22]. The increment could be attributed partly due to the release and dissemination of improved varieties developed through selection breeding that were adaptable to the local environment and also due to the awareness created on production and management of the crop [54].

During the last few decades under Ethiopian crop variety improvement programme, about 21 relatively high yielding genotypes of finger millet have been introduced in the cropping system to increase food production. National breeding program of finger millet had focused mainly on agronomic traits such as yields, drought tolerance and disease resistance [55] and none of the improved genotypes were evaluated for nutritional quality, especially micronutrient concentration. However, there will be unforeseen consequences which is changes from more diverse traditional cropping systems to mainly high yielding cereal production systems resulted in less micronutrient with lower bioavailability output of many farming systems and a rapid increase in micronutrient malnutrition (e.g., deficiencies of Fe, Zn and Ca) in resource-poor-families dependent on these agricultural systems for sustenance [56]. Therefore, it is imperative to assess the genotypic variation in nutritional quality (mineral and antinutritional concentrations, relative bioavailability of minerals) of released genotypes so far. Likewise, it is unequivocally important to investigate impact of agronomic biofortification on crop productivity, grain mineral concentration and their bioaccessability.

1.4 Research hypothesis

- Mineral and antinutrient concentration as well as relative bioavailability will varies as a result of genotypic difference among improved finger millet;
- Agronomic biofortification with Fe and Zn enhances the yield of finger millet and affected by genotype, slope position as well as location;
- Agronomic biofortification with Fe and Zn enhances the grain Fe and Zn concentration of finger millet and affected by genotype, slope position as well as location;
- Agronomic biofortification with Fe and Zn increase total daily absorbed zinc (TAZ) of 3 finger millet genotypes and affected by genotype;
- Bioaccessibilty of grain Zn and Fe will increases as a result of agronomic biofortification with Zn and Fe and traditionally fermentation.

1.5 Objectives

1.5.1 General objective

To evaluate the nutritional quality, investigate response to agronomic biofortification and bioaccessibility of different finger millet genotypes of Zn and Fe

1.5.2 Specific objectives

1. To assess the genotypic difference in mineral and antinutrient concentration as well as relative bioavailability of improved finger millet genotypes

2. To evaluate the impact of Fe and Zn agronomic biofortification on yield performance, grain Fe and Zn concentration and total daily absorbed zinc (TAZ) of finger millet genotypes as affected by genotype, slope position and location

3. To evaluate grain mineral bioaccessibilty of Zn and Fe biofortified finger millet.

1.6 Significance of the study

The current research output will help to promote finger millet production, utilization and marketing including export for its nutritional merits through the following points:

- For the genetic improvement of any crop plant, it is essential to know the nature and magnitude of variability present in base genotypes, hence, this project helps to understand the genotypic differences in nutritional quality among improved finger millet that has been released so far;
- Provide information on different genotypes of finger millet response to Zn and Fe agronomic biofortification towards yield as well as grain Fe and Zn concentration. This can be exploited in crop improvement, thereby serves as a platform for specific breeding objectives and helps policy makers nationally;
- Provide information on impact of Zn agronomic biofortification on grain phytate concentration and potential bioavailability Zn;
- Provide much needed information on finger millet minerals bioaccessibility and fermentation impact on it to the academic and research world, thereby, helps local consumers to attain dietary recommendations for minerals and at the same time can reduce the risk of side effect and toxicity associated with taking high dose of multiple micronutrient supplements;
- Provide insight on the effects of location and slope position on the effectiveness of Zn and Fe agronomic biofortification towards yield as well as grain Fe and Zn concentration.

1.7 Scope of the study

The scope of this study is to investigate multiple factors ranging from the impact of soil Fe and Zn deficiency amendment on crop Zn, Fe and antinutritional concentration as well as yield performance to the potential bioavailability of grain Fe and Zn in digestion system. It also discovers the genotypic difference in mineral and antinutrient concentration and the relative bioavailability of Fe and Zn of fifteen improved finger millet genotypes. The study also investigates the environmental factors like location and slope position on crop response to Fe and Zn agronomic biofortification towards yield as well as grain Fe and Zn concentrations. In addition the study explores impact of traditional fermentation on the potential bioavailability grain Fe and Zn.

1.8 Limitations of the study

The current study investigate the *in vitro* bioavailability of minerals following three step digestion at simulated physiological conditions. This technique can be useful to identify enhancers and/or inhibitors (phytate, oxalate, polyphenols and their degradation, ascorbic acid etc) but does not predict same magnitude of response as in humans; since small polyphenolic compounds and organic acid complexes is dialysable but not bioavailable and large molecules like ferritin can be absorbed but is not dialyzable [57]. Every results from *in vitro* studies on mineral bioaccessibility must always be confirmed with *in vivo* studies since it depends on multiple effects that could not be entirely addressed under any of the *in vitro* mineral bioavailability/bioaccessibility tests [57]. For example, transport rates in dialysis bag might be lower, less leaky, less discrimination on the basis of molecular size of compounds transported parallellary compared to the actual cells [58].

1.9 Structure of the dissertation

Chapter 1: General Introduction: "Effectiveness of agronomic biofortification strategy in fighting against hidden hunger" Published on *"Agronomy volume 13 issue 8"* as a special issue under the theme "*Importance of Zn Fertilization: Biofortification of Food Crops and Zoil Zn Status*". Journal impact factor: 3.7.

Chapter 2: "Genotypic variances in mineral concentration and relative bioavailability of improved finger millet in Ethiopia" Submitted to *Scientific reports* and under review as a special issue under the theme "*biofortification*".

Chapter 3: "Genotypic response of finger millet to zinc and iron agronomic biofortification, location and slope position towards yield" Published on "*Agronomy volume 13 issue 6"* as a special issue under the theme "*Transforming AgriFood Systems under a Changing Climate*". Journal impact factor: 3.949.

Chapter 4: "Impact of zinc and iron agronomic biofortification on grain mineral concentration of finger millet varieties as affected by location and slope" Published on "*Frontiers in nutrition volume 10"*. Journal impact factor: 6.59.

Chapter 5: "Agronomic biofortification of zinc fertilizer enhances the potential bioavailability of finger millet intrinsic zinc" to be submitted

Chapter 6: "Mineral bioaccessibilty of agronomically biofortified with zinc and iron and traditionally fermented finger millet" to be submitted

Chapter 7: General Discussion/Conclusions and Recommendations

1.10 Conference presentation

1. "*Agronomic biofortification improves the grain zinc and iron concentration of Ethiopian finger millet varieties: suitable approach to fight hidden hunge*." Presented at Agriculture, Nutrition and Health Academic week, 25 June – 1 July 2023, held at Lilongwe, Malawi.

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Chapter 2: Differences in the nutritional quality of improved finger millet genotypes in Ethiopia

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Abstract

Improved crop genotypes are constantly introduced**.** However, information on their nutritional quality is generally limited. The present study reports the proximate composition and mineral concentrations and their relative bioavailability of improved finger millets of different genotypes. Grains of finger millet genotypes (n=15) grown in research station during 2019 and 2020 in Ethiopia, and replicated three times in a randomized complete block design, were analysed for proximate composition, mineral concentration (iron, zinc, calcium, selenium), and antinutritional factors (phytate, tannin and oxalate). Moreover, the antinutritional factors to mineral molar ratio method was used to estimate mineral bioavailability. The result shows a significant genotypic variation in protein, fat and, fibre, ranging from 10 to 14.6%, 1.0 to 3.8%, and 1.4 to 4.6%, respectively. Similarly, different finger millets genotypes had significantly different mineral concentrations ranging from 3762 ±332 to 5893 ±353 mg kg^{-1} for Ca, 19.9 ±1.6 to 26.2 ±2.7 mg kg^{-1} for Zn, 36.3±4.6 to 52.9±9.1 mg kg^{-1} for Fe and 36.6 ±11 to 60.9 ±22 µg kg^{-1} for Se. Phytate (308-360 µg g^{-1}), tannin (0.15-0.51 mg g^{-1}) and oxalate (1.26-4.41 mg g^{-1}) concentrations were also influenced by genotype. Antinutritional factors to minerals molar ratio were also significantly different by genotypes but were below the threshold for low mineral bioavailability. Genotype significantly influenced mineral and antinutritional concentrations of finger millet grains. All finger millet genotypes possess good mineral bioavailability. The high Ca concentration in finger millet, compared to in other cereals, could play a vital role to combating Ca deficiency. The result suggests the different finger millet

genotypes possess good nutrient content and may contribute to the nutrition security of the local people.

2.1 Introduction

Finger millet (*Eleusine coracana* L.) represents one of the critical plant genetic resources for food security of populations from arid, infertile and marginal lands [1]. In the semiarid tropics of Eastern Africa, finger millet is the major staple food for millions of resource poor people [2]. Finger millet is adaptable to adverse agro-ecological conditions with minimal agricultural inputs (fertilizer, pesticides, and herbicides). It is also disease tolerance and is productive on marginal land where other crops cannot be grown [3, 6].

Global finger millet production is not known because this crop has typically grouped and reported with other millets. Total production of millet is about 34 million tonnes worldwide [5] and is estimated to represents about 12.8% (4.3 million tonnes) of all millet crops production [6]. In Ethiopia, finger millet is the sixth most important crops after teff (*Eragrostis tef* Zucc.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), and barley (*Hordeum vulgare* L.). An estimated 1.2 million tonnes of finger millet is produced in Ethiopia on 48 thousand hectares of land. [7]. Nationwide, 1.5 million households are directly engaged in finger millet production. The production has increased by 300% in the previous 20 years [7].

During the last few decades, about 21 relatively high yielding genotypes of finger millet have been introduced in the cropping system, under the Ethiopian crop variety improvement programme. The national breeding program of finger millet has focused mainly on agronomic traits such as yields, drought tolerance and disease resistance [8]; however, there is limited information on nutritional quality. The present study evaluated nutritional quality of finger millet grains of different genotypes in Ethiopia. Information on the nutritional quality of finger millet will help to design an advocacy work to increase its consumption and agricultural interventions to increase nutrient content on the edible portion of the crop.

2.2 Results

Finger millet genotypes showed significant ($p < 0.01$) variation in mineral concentrations (Table 1). Greater variability was observed in Ca concentration ranging from 3540 (Axum) to 6117 (BKFM0010) mg kg^{-1}. Bereda and BKFM0010 genotypes had the highest Ca concentration while Axum, Wama, Boneya, Bako-01 and Gudetu genotypes had the lowest Ca concentration (Table 2.1).

Table 2.1. Mineral concentrations (mg kg^{-1}) of finger millet from Ethiopia as affected by genotypes

Genotype	Fe	Zn	Ca	Se (µg kg^{-1})
Addis-01	47.7 ± 10.1^{ab}	22.2 ± 2.5^{abcd}	5140 ± 429^{gh}	52.1 ± 19^{ab}
Axum	36.3 ± 4.6^{a}	19.9 ± 1.6^{a}	3762 ± 332^{a}	36.9 ± 9^{a}
Bako-09	49.9 ± 13.4^{b}	20.6 ± 4.1^{ab}	3994 ± 231^{abc}	45.1 ± 9^{ab}
Bereda	44.3 ± 5.5^{ab}	24.4 ± 1.9^{cde}	5504 ± 412^{hi}	51.5 ± 18^{ab}
BKFM0010	48.2 ± 4.5^{ab}	25.5 ± 3.4^{de}	5893 ± 353^{i}	60.9 ± 22^{b}
Boneya	44.7 ± 12.3^{ab}	21.4 ± 1.8^{abc}	3977 ± 397^{abc}	36.6 ± 11^{a}
Diga-01	52.9 ± 9.1^{b}	21.9 ± 1.8^{abcd}	5193 ± 371^{gh}	50.4 ± 15^{ab}
Gudetu	48.6 ± 10.6^{ab}	21.7 ± 2.6^{abcd}	4237 ± 550^{abcd}	52.4 ± 18^{ab}
Gute	41.4 ± 7.5^{ab}	20.3 ± 1.6^{ab}	4315 ± 473^{bcde}	43.5 ± 14^{ab}
Meba	51.3 ± 10.9^{b}	23.4 ± 2.5^{bcde}	5008 ± 419^{fgh}	48.7 ± 15^{ab}
Paddet	48.7 ± 6.6^{b}	26.2 ± 2.7^{e}	4460 ± 381^{cde}	43.6 ± 14^{ab}
Tadesse	47.4 ± 5.8^{ab}	25.3 ± 2.9^{de}	4572 ± 311^{def}	42.8 ± 12^{ab}
Tesema	49.1 ± 7.7^{b}	24.7 ± 3.4^{cde}	4312 ± 259^{bcde}	41.1 ± 10^{ab}
Urji	49.7 ± 7.2^{b}	23.9 ± 2.6^{bcde}	4783 ± 395^{efg}	47.7 ± 16^{ab}
Wama	43.0 ± 9.6^{ab}	20.4 ± 4.2^{ab}	3782 ± 382^{ab}	37.1 ± 13^{a}

Significance at the $p < 0.01$ are represented with different letters

Finger millet Zn concentration was significantly different ($p<0.001$) between genotypes. Paddet and Axum genotypes showed the highest and the lowest grain Zn concentration, respectively (Table 2.1). Similarly, there was strong genotype influence on grain Fe concentration while moderate evidence ($p <0.01$) was observed on grain Se concentration (Supplementary material). Irrespective of locations the analysis of variance indicated that there was significant difference ($p < 0.001$) in antinutritional concentration among genotypes (Supplementary material).

Phytic acid concentration of studied finger millet genotypes (Table 2.2). Urji genotype showed significantly greater phytic acid and tannin concentrations. On the other hand, Addis-01, Boneya, Gute, Diga-01, Paddet and Gudetu genotypes had the lowest phytic acid concentrations (Table 2.2). In addition, Boneya, Diga-01, Gudetu, Gute and Meba genotypes had the lowest tannin concentrations (Table 2.2).

Table 2.2. Antinutritional concentration of finger millet from Ethiopia as affected by genotypes

Genotype	Phytate (µg g^{-1})	Tannin (mg g^{-1})	Oxalate (mg g^{-1})
Addis-01	311.5±2.9^{a}	0.32^{d}	3.39±0.3^{d}
Axum	319.6±1.3^{abcde}	0.25^{bc}	3.15±0.6^{cd}
Bako-09	330.6±3.2^{cdef}	0.31^{d}	2.21±0.3^{ab}
Bereda	329.3±2.9^{bcdef}	0.28^{c}	3.31±0.2^{d}
BKFM0010	348.5±3.2^{gh}	0.23^{b}	1.81±0.4^{ab}
Boneya	313.5±5.2^{ab}	0.17^{a}	3.39±0.8^{d}
Diga-01	317.1±3.2^{abcd}	0.17^{a}	1.89±0.3^{ab}
Gudetu	323.5±3.6^{abcde}	0.17^{a}	1.34±0.2^{a}
Gute	314.3±4.8^{abc}	0.16^{a}	2.52±0.3^{bcd}
Meba	330.6±4.3^{def}	0.17^{a}	2.21±0.3^{ab}
Paddet	323±6.8^{abcde}	0.24^{b}	2.52±0.3^{bcd}
Tadesse	342.8±2.6^{fgh}	0.23^{b}	2.37±0.2^{bc}
Tesema	341.4±19.9^{fgh}	0.24^{b}	2.52±0.3^{bcd}
Urji	349.2±3.5^{h}	0.50^{e}	1.89±0.3^{ab}
Wama	334.8±4.5^{efgh}	0.24^{b}	2.21±0.4^{ab}

Significance at the $p < 0.001$ are represented with different letters.

Note: The upper limit for intake of the antinutritional factors is 0.6 mg/kg of body weight for tannin [9] and 50 mg/day of oxalate [10].

Molar ratios of anti-nutritional factors to mineral concentration as proximate indicator for bioavailability are presented in Table 3. The molar ratio of phytate to Fe, phytate to Zn, phytate to Ca, phytate × Ca to Zn and oxalate to Ca was in the range of 0.51 - 0.71, 1.22 - 1.63, 0.004 - 0.005, 0.14 - 0.2 and 0.14 - 0.39, respectively (Table 2.3).

Table 2.3. Molar ratio of phytate to iron, zinc, calcium and oxalate to calcium

Genotype	Phytate:Fe	Phytate:Zn	Phytate:Ca	Phytate x Ca:Zn	Oxalate:Ca
Addis-01	0.55	1.39	0.004	0.18	0.30
Axum	0.74	1.59	0.005	0.15	0.38
Bako-09	0.56	1.59	0.005	0.16	0.25
Bereda	0.63	1.34	0.004	0.18	0.27
BKFM0010	0.61	1.35	0.004	0.20	0.14
Boneya	0.59	1.45	0.005	0.14	0.39
Diga-01	0.51	1.43	0.004	0.19	0.17
Gudetu	0.56	1.48	0.005	0.16	0.14
Gute	0.64	1.53	0.004	0.17	0.27
Meba	0.55	1.40	0.004	0.18	0.20
Paddet	0.56	1.22	0.004	0.14	0.26
Tadesse	0.61	1.34	0.005	0.15	0.24
Tesema	0.59	1.37	0.005	0.15	0.27
Urji	0.59	1.45	0.004	0.17	0.18
Wama	0.66	1.63	0.005	0.15	0.27

Cut off values: Phytate:Zn >15, phytate:Fe >1, phytate:Ca > 0.24, phytate × Ca:Zn >200, and oxalate:Ca >1

Protein content of finger millet shows variation between genotypes (Table 2.4). Paddet shows significantly higher protein whereas BKFM0010, Gute, Bako-09 and Diga-01 varieties shows lower protein content. Variation in crude fibre content of finger millet varieties ranges from 1.44 to 4.63 % (Table 2.4). BKFM0010 and Bako-09 varieties possess significantly higher and lower crude fibre content among the varieties, respectively. Total lipid and minerals also ranges from 1.05 to 3.81 % and from 1.01 to 3.97 % between the genotypes of finger millet, respectively (Table 2.4). The highest crude fat content was found in Gute while Paddet, Urji and Diga-01 shows significantly highest total mineral content. Variation in carbohydrate was ranging from 76.7 to 84.0% is observed where Bako-09 and Axum shows significantly highest carbohydrate content (Table 2.4).

Table 2.4. Proximate composition (g 100g^{-1} in dry base) of different finger millet genotypes

Variety name	Protein	Crude fibre	Crude fat	Total ash	Carbohydrate
Addis-01	11.85 ± 0.53^{cd}	2.06 ± 0.04^{b}	1.1 ± 0.02^{a}	2.7 ± 0.25^{d}	82.3 ± 0.51^{hi}
Axum	11.68 ± 0.11^{bc}	3.19 ± 0.12^{d}	1.09 ± 0.02^{a}	1.1 ± 0.08^{a}	82.9 ± 0.14^{ij}
Bako-09	11.09 ± 0.42^{abc}	1.5 ± 0.05^{a}	1.07 ± 0.02^{a}	2.69 ± 0.1^{d}	83.7 ± 0.35^{j}
Bereda	12.99 ± 0.13^{e}	2.3 ± 0.06^{c}	1.9 ± 0.5^{bc}	2.13 ± 0.01^{c}	80.7 ± 0.35^{ef}
BKFM0010	10.56 ± 0.41^{a}	4.58 ± 0.06^{g}	1.67 ± 0.02^{b}	3.32 ± 0.08^{e}	79.9 ± 0.46^{de}
Boneya	12.95 ± 0.39^{e}	3.06 ± 0.08^{d}	2.15 ± 0.06^{c}	3.23 ± 0.07^{e}	78.6 ± 0.54^{b}
Diga-01	11.32 ± 0.39^{abc}	2.19 ± 0.03^{bc}	1.63 ± 0.07^{b}	3.78 ± 0.03^{f}	81.1 ± 0.33^{fg}
Gudetu	13.02 ± 0.11^{e}	3.17 ± 0.09^{d}	2.68 ± 0.1^{d}	2.17 ± 0.04^{c}	79 ± 0.32^{bc}
Gute	11.07 ± 0.26^{abc}	2.13 ± 0.11^{bc}	3.77 ± 0.05^{f}	1.11 ± 0.03^{a}	81.9 ± 0.31^{gh}
Meba	11.5 ± 0.37^{bc}	3.8 ± 0.07^{f}	2.17 ± 0.1^{c}	1.61 ± 0.14^{b}	80.9 ± 0.11^{f}
Paddet	14.15 ± 0.58^{f}	3.49 ± 0.09^{e}	1.68 ± 0.12^{b}	3.74 ± 0.19^{f}	76.9 ± 0.29^{a}
Tadesse	12.97 ± 0.23^{e}	3.24 ± 0.1^{d}	3.21 ± 0.08^{e}	3.24 ± 0.1^{e}	77.4 ± 0.25^{a}
Tesema	12.71 ± 0.5^{de}	3.18 ± 0.21^{d}	2.64 ± 0.07^{d}	1.6 ± 0.02^{b}	79.9 ± 0.15^{de}
Urji	11.46 ± 0.32^{bc}	3.12 ± 0.03^{d}	2.18 ± 0.06^{c}	3.75 ± 0.09^{f}	79.5 ± 0.43^{cd}
Wama	10.84 ± 0.31^{ab}	3.55 ± 0.08^{e}	1.62 ± 0.07^{b}	1.6 ± 0.09^{b}	82.4 ± 0.3^{hi}

Significance at the $p < 0.05$ are represented with different letters

2.3 Discussion

Finger millet plays a major role in food and nutrition security for millions of resource poor smallholding farming communities [2]. Breeding programs often focus on agronomic traits such as yield, drought tolerance and disease resistance [8]. In addition, it is crucial to understand nutritional quality prior to genotype verification and seed release. The present study evaluated the nutritional quality (nutrient content, mineral relative bioavailability and antinutritional factors) of different genotypes of finger millet grains.

The present study revealed that finger millet genotypes are rich source of Ca. Sharma *et al.,* [11] and Kumar *et al.,* [12], also reported wide variation in finger millet Ca concentration in the range of 530 and 4540 mg kg^{-1} (n = 202 genotypes) and 720 to 4520 mg kg^{-1} (n = 113 genotypes), respectively. Similarly, finger millet Ca concentration ranges from: 1620 to 4870 mg kg^{-1} (n = 36 genotypes) [13], 1505 to 4528 mg kg^{-1} (n = 26 genotypes) [14], 2766 to 3319 mg kg^{-1} (n = 5 genotypes) [15], 3341 to 3540 mg kg^{-1} (n = 3 genotypes) [16], and 3180 to 6590 mg kg^{-1} (n = 12 cultivars) [17]. Varied concentration of Ca in finger millet was also reported by Patil *et al.,* [18], ranging between 9000 and 14000 mg kg^{-1} (n = 37 genotypes).

Previous studies partly associate variation in mineral accumulation in grains to specific genes in the plant. For example, Mirza *et al.* [19] and Sharma *et al.* [11] reported that *EcCBP* and *EcCIPK7* genes and the activities of CaX exchanger and calmodulin (CAM) proteins in finger millet resulted high Ca accumulation. They also reported that these two genes were highly expressed in high Ca genotypes compared to medium and low Ca genotypes [12, 20].

The current study revealed that finger millet Ca concentration is about 400% greater than that of other millets (Pearl, Proso, Foxtail, and Kodo) and 90% to 230% greater than other cereals such as wheat, barley, sorghum, maize, rice, and rye [21]. Genotypic variation in Zn concentration is in agreement with other similar studies. Puranik *et al.*, [22] reported finger millet Zn concentration between 10.2 and 26.6 mg kg^{-1} (n = 48 genotypes). Singh and Srivastava, [23] and Panwar *et al.*, [15] also experimented on finger millet and reported variation in Zn concentration ranging from 9.2 to 25.5 mg kg^{-1} (n = 16 genotypes) and 20 to 29.6 mg kg^{-1} (n = 5 genotypes), respectively.

Many reports on the genotypic variation in Fe concentrations in finger millet indicates concentrations in the range of 18.0 - 166.0 mg kg^{-1} (n = 106 genotypes) [16-18, 22, 24]. Similarly, Fe concentrations in the current study grain samples fall within this range. In addition, variation in Se concentration as a result of genotypic differences are in agreement with those observed by Udeh *et al.*, [25] who found significant variation in Se concentration in the range 20 to 50 µg kg^{-1}.

High accumulation of Fe and Zn in finger millet grains has been attributed to the regulation of potential key regulatory genes involved in Fe and Zn homeostasis particularly *EcFER1, EcIRT2*, *EcYSL2*, *EcZIP1* and *EcZTP29* genes [26]. Similarly, high concentrations of Se in finger millet has been also attributed to regulatory genes involved in Se homeostasis such as HOX4 and SPL genes [27].

The analysis of variance indicates significant variation in phytic acid concentrations that are compared to those reported by Nakarani *et al.,* [28] which were in the range of 2108 to 3028 µg g^{-1} (n = 10 genotypes). Other studies also reported very wide variation (3363 to 14020 µg g^{-1}) in phytic acid concentration of finger millet [25, 29]. The variation in current and previous studies is possibly influenced by genotype, soil and climatic factors, and crop growing season

[30]. Phytate is the major storage (up to 82%) form of P in plant [31] and every factor that affects plant P uptake also affects grain phytic acid concentration. For example, P become unavailable to plants in both acidic and alkaline soils. However, pH 6 to 7 are reported to be optimum pH for P absorption. On the other hand, P is subjected to iron and aluminium fixations at lower pH and by Ca at higher pH [32]. Both plant growth and P uptake are slow in the winter and release of P from organic matter is apparently slow while in summer there is further decomposition of organic residues brings an increase in anion-exchangeable P and in soil P become soluble [33].

Tannin concentrations were also lower than previously reported values. Nakarani *et al.*, [28] and Shibairo *et al.*, [24] reported values ranging between 3.4 and 5 mg g^{-1} (n = 10 genotypes) and 2.76 and 5.4 mg g^{-1} (n = 6 genotypes), respectively. Another study also reported a value of 1.64 ± 0.01 mg g^{-1} tannin concentration for finger millet [29]. The reason for lower tannin concentration in this study, besides genetic factors, could be attributed to the crop growing temperature which has been reported to influence the tannin concentration in cereals [34], higher temperature might result in higher tannin concentration [24].

Similar studies on oxalate concentrations in finger millet reports values ranging from 0.2 to 0.26 mg g^{-1} (n = 10 genotypes) [28]. The present study shows higher oxalate concentration which could arise for many reasons including: synergetic and antagonistic effect on oxalate from N and P, respectively [35] and season/temperature also reported to influence oxalate accumulation [36]. For example, when nitrate is reduced hydroxyl ions (OH^-) are produced and the increased levels of OH^- may serve as a signal triggering the organic acid biosynthesis like oxalic acid to neutralize the excess levels of OH^- [37]. There are more favourable growth factors prevailing in spring season which help higher metabolic rate of the younger tissues to synthesize oxalate [36].

Phytates inhibit Fe, Zn and Ca absorption in to the human body system. Oxalate also inhibits Ca absorption by forming insoluble and indigestible complexes and additionally Ca competitively inhibits Zn absorption. The amount of these complexes and the molar ratio of phytate and oxalate to minerals will therefore affect bioavailability [38, 39]. The molar ratio of phytate to mineral among the finger millet genotypes studied shows that they are all less than cut-off values phytate:Zn is > 15 for low bioavailability, 5-15 for medium bioavailability and < 5 for high bioavailability [40]. The molar ratio of phytate x Ca:Zn of all finger millet genotype in a current study was substantially lower than the cut-off values of 200 [41] suggesting that the finger millet varieties in the present study possess bioavailable Zn. The molar ratio of phytate:Fe, phytate:Ca and oxalate:Ca were lower than the cut-off values of >1 [42], > 0.24 [43] and >1 [40], respectively. All the finger millet genotypes had lower molar ratio suggesting good bioavailability of Fe and Ca.

The present result is in agreement with similar previous experiment from Ethiopia that reports 6.3 to 10.5 % (n = 3 genotypes, 6 cultivars) variation in crude protein content [8]. Another study on finger millet indicates variation in crude protein content between 6.7 and 12.3 % (n =36 genotypes) [13]. Puranik *et al.,* [22], also analysed finger millet from East Africa and reported wide variation in crude protein ranging from 3.9 to 11.3 % (n = 48 genotypes).

Carbohydrate content of finger millet in the present study shows variation between 76.7 and 84.1%. Previous study conducted on finger millet reported that carbohydrate varies from 84.7 to 86.6 % (n = 2 genotypes) [44]. Similar research on finger millet from Sri Lanka indicated variation in carbohydrate from 86.6 to 87.3% (n = 3 genotypes) [16]. Patil *et al.*, [18], Nakarani *et al.*, [28] and Shibairo *et al.*, [24], reported variation in carbohydrate content which ranges between 68.2 and 76.4 % (n = 37 genotypes) and 71.9 and 76.4 % (n = 10 genotypes), 75.6 and 78.5 % (n = 6 genotypes), respectively.

Crude fibre content of finger millet genotypes ranges from 1.44 to 4.63 % in present study. The present study is in agreement with Kaur *et al.*, [17], which reported that variation in crude fibre content of finger millet ranging between 3.2 and 5.8% (n = 12 cultivars). Patil *et al.*, [18] and Nakarani *et al.*, [28], also reported variation of finger millet's crude fibre ranging from 3.7 to 4.2 % (n = 37 genotypes) and from 3.1 to 3.8 % (n = 10 genotypes), respectively. Similarly, previous studies reported variation in crude fibre content of different finger millet genotypes ranging from 3.1 to 5.6 % [34, 49].

Current experiment shows that the total lipid ranged from 1.1 to 3.8 % between finger millet genotypes. Similar previous studies also reported wide variation in total lipid of finger millet from all over the world that ranges from 0.3 to 4.1 % (n = 45 genotypes) [17, 18, 24, 28, 29, 44].

Total mineral content significantly varies between finger millet genotypes and ranges from 1.0 to 4.0 %. Similar study on finger millet from Ethiopia shows 1.7 to 3.4 % (n = 3 genotypes, 6 cultivars) variation in total mineral content [8]. Different researches around the globe shows that genotypically finger millet varies in their total mineral content, between 1.5% and 3.6% [16, 17, 23, 44].

2.4 Conclusion

Our study shows that finger millet proximate composition, mineral and antinutrient content as well as mineral bioavailability significantly vary by genotype. The present study finger millet genotypes in general are good sources of Ca and protein, and a fair source of Fe and Zn. Moreover, all finger millet genotypes in present study exhibited excellent Zn, Fe and Ca bioavailability. Specifically, Bereda and BKFM0010 genotypes can be suggested for their highest mineral concentration and Paddet genotype for its highest protein in future breeding programmes. The highest concentration and relative bioavailability of Ca in finger millet could play a role in combating preeclamsia which is the second most cause of maternal mortality and Ca deficiency is the major factor of its occurrence [45]. Genotype, perhaps, significantly influences the minerals and anti-nutritional concentrations of finger millet. Even though finger millet has a high nutrient quality, use of finger millet in the daily diet is low [46], suggesting the need for community nutrition education on the promotion of the nutritional benefit of finger millet and product development. Further investigations focusing on *in vivo* bioavailability testing of finger millet minerals are also strongly recommended.

Study strength and limitation

Strength: the current study uses 15 out of 21 improved finger millet genotype for experiment, all the field and laboratory experiments were replicated three times, about 270 finger millet samples were analysed in the laboratory, field experiments were repeated for two seasons and two locations, about 11 parameters were analysed in the laboratory.

Limitation: the current study uses the molar ratio of antnutrients to mineral to estimate bioavailability of minerals. This method is a proxy indicator for minerals bioavailability but does not predict same magnitude of response as in humans.

2.5 Material and method

2.5.1 Field experiment

Out of a total of 21 improved new finger millet genotypes, 15 genotypes were obtained from seed maintainers (Dagi-01, BKFM0010 black grain colour, Urji white grain colour, Addis-01, Axum, Bako-09, Bereda, Boneya, Gudetu, Gute, Meba, Paddet, Tadesse, Tessema, Wama brown grain colour). Genotypes were improved for agronomic traits like high yield, disease resistance and stability [8]. Genotypes that are suitable for midland were selected for this study. The finger millet genotypes were grown in a randomized completed block design (RCBD) in field experiments at research stations, in two locations: Bako Agricultural Research Centre (9º

91’ 831”N 37 º 42’ 492”E) and Gute sub site (9º 00’ 536”N 36 º 38’ 243”E) to study the influence of genotype variability on concentration and bioavailability of minerals over two seasons (during 2019 and 2020). Both sites are characterized as sub-humid midlands located between 1600–2300 meters above sea level (masl) and receive an average annual rainfall of 800–1200 mm [47].

2.5.2 Agronomic management

The plot size was 3m x 3m, with gangway between plots being 1m while distance between block and the border were 0.5m each. The experiment was repeated in two growing seasons; 2019 and 2020. Seed was sowed in July and harvested in November. Planting was carried out by hand drilling at a seed rate of 15kg ha^{-1}. Each experimental plot had 40cm inter-row spacing. Fertilizers, NPS (131 kg ha^{-1}) was applied at sowing and urea (54 kg ha^{-1}) was applied after 45 days at first weeding. Each plot was weeded at least six times by hand and no pesticide or herbicides was applied.

2.5.3 Sample collection and preparation

After obtaining permission from Ethiopian Agricultural Research Institute, crop samples were collected from the farm and prepared in the laboratory following the method as described in Gashu *et al.*, [48]. Briefly, matured and dried finger millet crop fingers were collected from each plot using scissors. The crop samples were hand threshed in the laboratory to produce approximately 1 kg of grain before whole-grain samples were packed in paper bags and allowed to air dry. The grain samples were then ground using a stainless-steel coffee grinder, which was wiped clean before use and after each sample with a non-abrasive cloth. All preparations were done away from sources of soil and dust contamination. A 20 g subsample (following a representative coning and quartering system) of the ground finger millet was shipped to the University of Nottingham, UK for mineral analysis. The use of plants in the present study complies with international guidelines.

2.5.4 Mineral analysis

Ground finger millet grain samples were acid digested in a hot plate as described in Gashu *et al.*, [48]. Briefly, about 0.2 g of sample was weighed into digestion tubes and placed into a heating block (Multicube 48, Anton Paar Ltd, UK). Concentrated HNO_3 (8 mL, trace metal grade, Fisher Chemical, USA) was added to each tube and left for 30 minutes at room temperature. The samples were then heated for 2 hours at 115°C and left to cool before dilution to 50 mL using MilliQ water (18.2 MΩ cm; Fisher Scientific). A further 1 in 10 dilution was undertaken immediately prior to analysis by inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo Fisher Scientific, Bremen, Germany). A certified reference material (CRM, Wheat 1567b, National Institute of Standards and Technology, Gaithersburg, MD, USA) was used to determine % recovery. Operational blanks (n=20) were analysed at the same time to determine the limit of detection (LOD) for each element.

2.5.5 Anti-nutritional factors analysis

Phytic acid was analysed using the Wade Reagent method, after Latta & Eskin [49] and modified by Vaintraub & Lapteva [50]. For extraction of phytic acid 0.2 g flour samples were centrifuged at 3000 rpm for 1 hour after adding 10 mL of 0.2 M HCl. Then 3 mL of the supernatant and 2 mL of Wade solution were added and samples shaken to mix. Absorbance was measured at 520 nm using a UV-VIS spectrophotometer (Lambda 950, PerkinElmer, Waltham, USA) and the amount of phytic acid calculated and expressed in µg g^{-1}.

Oxalate in the grain flour samples was determined following the method of the Association of Official Analytical Chemists [51]. Briefly, 1 g of sample was weighed into a 100 mL conical flask before 75 mL of 3 M H_2SO_4 was added and the solution mixed for about 1 hour before filtering. The filtrate was collected and titrated against hot (80– 90°C) 0.1 M $KMnO_4$ solution to the point when a faint pink color appeared that persisted for at least 30 seconds. The concentration of oxalate in each sample was obtained using the assumption that 1 mL 0.1 M $KMnO_4$ = 0.006303 g oxalate [51].

Tannin was determined using the vanillin-HCl assay method [52, 53]. Briefly, 10 mL of 1 % HCl in methanol was added to 1 g grain flour in a screw capped test tube and placed on a mechanical shaker for 24 hours at room temperature. The tube was centrifuged at 1000 g for 5 minutes and 1 mL of supernatant was removed and mixed with 5 mL of vanillin-HCl reagent. Absorbance at 500 nm was measured after 20 minutes.

2.5.6 Phytate and oxalate to mineral molar ratio calculation

The inhibitory effect of dietary phytate on the bioavailability of Fe, Zn and Ca, and oxalate on Ca bioavailability was determined through calculation of molar ratios (phytate:Fe, phytate:Zn, phytate x Ca:Zn and phytate:Ca and oxalate:Ca) and the millimoles used were 660 mg/mmol for phytate, 55.845 mg/mmol for Fe, 65.4 mg/mmol for Zn, 40 mg/ mmol Ca and 88.019 mg/mmol for oxalate [38, 39]. Phytate:Zn >15 [40], phytate:Fe >1 [42], phytate:Ca > 0.24 [43], phytate × Ca:Zn >200 [40], and oxalate:Ca >1 [40] were used as cut-offs. Samples with molar ratio values higher than the cut-off values were considered less bioavailable.

2.5.7 Proximate composition analysis

2.5.7.1 Crude protein

Crude protein content of samples was quantified by Kjeldahl methods [51]. Briefly, 0.5 g of powder sample was weighed into tecator tube and digested by heating at 370ºC for 3 hours in the presence of 6 mL mixed sulfuric acid (H_2SO_4), 3.5 mL hydrogen peroxide (H_2O_2), 3 g of catalyst mixture: potassium sulphate (K_2SO_4) and copper sulphate ($CuSO_4$). After digestion was completed, the clear solution was cooled for 30 minutes. After cooling, it was distilled by steam distillation with 25 mL of 40 % of sodium hydroxide (NaOH) and the ammonium is released as a form of ammonia (NH_3). Finally, the condensed NH_3 is trapped by 1 % boric acid and titrated by 0.1N hydrochloric acid (HCl). The nitrogen content was estimated by titration of the borate anion formed with 0.1N HCl. The amount of Nitrogen was calculated using the following equation:

Equation 1: Nitrogen % = (V x N x 14 x 100) / (1000 x Wo)

Where; V- Volume of HCl consumed to the end point of titration, N- The normality of the HCl used, Wo- Sample weight on dry matter basis, 14- The molecular weight of the atomic nitrogen

Equation 2: Protein % = Nitrogen % x 5.54

2.5.7.2 Crude fat

Crude fat was determined by Soxlet extraction method [51]. Extraction cylinders were measured (W1) after cleaned and dried in oven at a 105°C for 1 hour. The bottom of the extraction thimbles were covered with a layer of fat free cotton and approximately 2 g of powder samples were measured in thimbles and covered with cotton layer (W). The thimbles were put in the extraction chamber. Extraction cylinders were filled with 50 ml of ether and moved into the heating plank. The extraction was run for about 4 hours and then the extraction cylinders were disconnected and put in a drying oven at 70°C for about 30 minutes. The cylinders were taken out of the oven and cooled in a desiccator for 30 minutes and the weight of cylinders were measured (W2). Finally the fat content of the samples were determined using the following equation:

Equation 3: Crude fat % = [(W2-W1)/W] x 100

2.5.7.3 Crude fibre

Two crucibles were cleaned and dried with 1 g celite in an oven at 105°C for 1 hour. Approximately 1 g of flour sample was weighed into pre-dried crucible (W1). Then, 200 mL of 1.25 % H_2SO_4 was added to each crucible and left to boil for 37 minutes. The acid was drained using a vacuum pump after 37 minutes and the samples were cooled for 5 minutes, washed with distilled water. Then, 200 mL of 1.25 % NaOH solution was added into each crucible and let to boil for 37 minutes. The base was drained using a vacuum pump and washed with distilled water. Crucibles containing residue were dried at 130ºC for 2 hours and cooled in a desiccator and weighed (W2). The residues were ashed in a muffle furnace at 550ºC for 3 hours and left to cool down to below 250ºC before removing from the furnace. The crucibles were cooled in a desiccator to room temperature and their weight were measured using analytical balance (W3) [51].

Equation 4: Crude fibre % = [(W2-W3)/W1] x100

2.5.7.4 Total ash

Total ash content was determined by following a method as described by Association of Official Analytical Chemists, [51]. Briefly, porcelain crucibles were cleaned, dried and their weight were measured (M1) after cooled in a desiccator for 30 minutes. Approximately 2.5 g of flour sample were measured in each crucible (M2) and charred on a hot plate under a fume hood until the smoke ceased down. Then the samples were ashed in muffle furnace at 550°C for 5 hours and left to cool down to below 250ºC before removing from the furnace. The crucibles were cooled in a desiccator to room temperature and their weight were measured (M3) and the ash content were determined by using the following equation:

Equation 5: Total ash = [(M3-M1) / (M2-M1)] x 100

2.5.8 Statistical analysis

Data were analysed using SPSS software version 20. The data are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was calculated to compare nutrient levels and concentrations of antinutritional factors across finger millet genotypes. Genotype was treated as fixed effect whereas block within farm and location were treated as random effects. The variance component for random effects was checked to get an idea of how important they might be relative to each other. $p< 0.001, 0.01, 0.05$ was considered significant.

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Author contributions

D.T, D.G, E.J.M.J, T.A, M.R.B conceptualize the research; D.T collected the samples, analysed the samples and wrote the original draft; D.T, E.H.B, L.W prepared and analysed the samples; writing—review and editing— D.T, D.G, E.J.M.J, E.H.B, L.W, T.A, M.R.B wrote, reviewed and edited the paper. All authors have read and agreed to the published version of the manuscript.

Data availability

The data generated in this study is available upon request from the corresponding author.

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Conflict of interest

The authors declare no conflict of interest

Supplementary material

Supplementary table 1: Analysis of variance of the effect of finger millet genotype on mineral concentration

	Sum of Squares	Mean Square	Df	F	Sig.
Fe	3053.161	218.083	14	2.825	.001**
Zn	748.590	53.471	14	7.229	.000**
Ca	69304620.088	4950330.006	14	32.998	.000**
Se	.008	.001	14	2.604	.002*

Significance code: ** < 0.001; * < 0.01

Supplementary table 2: Analysis of variance on effect of finger millet genotype on finger millet antinutritional concentration

	Sum of Squares	Mean Square	Df	F	Sig.
Phytate	8938.665	638.476	14	15.610	.000**
Tannin	.422	.030	14	193.473	.000**
Oxalate	21.807	1.558	14	12.375	.000**

Significance code: ** < 0.001

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Chapter 3: Genotypic response of finger millet to zinc and iron agronomic biofortification, location and slope position towards yield

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Abstract

The present study aimed to investigate the influence of genotypic differences on responses to zinc and iron agronomic biofortification among yields of finger millet. A field experiment was conducted over two seasons in farmers' fields in Ethiopia (2019, 2020). The experimental design had 15 treatment combinations comprising three finger millet genotypes and the applications of different combinations of zinc and iron mineral fertilizers. Five soil-applied fertilizer treatments (20 kg h^{-1} $FeSO_4$ + 25 kg h^{-1} $ZnSO_4$ + NPKS, 25 kg ha^{-1} $ZnSO_4$ + NPKS, 20 kg ha^{-1} $FeSO_4$ + NPKS, NPKS, and 30% NPKS) at two locations (Gojjam and Arsi Negelle, Ethiopia) and using two slope positions (foot and hill) were replicated four times in a randomized complete block design. Grain yield and biomass were evaluated on a plot basis. Plant height, total and productive tiller number, finger length of the longest spike and number of fingers per main ear were measured at the maturity stage. The combined soil application of $FeSO_47H_2O$ and $ZnSO_47H_2O$ increased the yield of the Meba genotype by 51.6%. Additionally, $ZnSO_47H_2O$ fertilizer application increased the yield of the Urji genotype by 27.6%. A yield enhancement of about 18.3% of the Diga-01 genotype was achieved due to the $FeSO_47H_2O$ fertilizers' application. The findings of the present study suggest that the influence of Zn and Fe agronomic biofortification on the yield of finger millet could be affected by genotype differences and environmental conditions.

Keywords: agronomic biofortification; finger millet; genotype; iron; yield; zinc

3.1 Introduction

Cereal crops naturally have very low grain zinc (Zn) and iron (Fe) concentrations, and growing them on soils deficient in potentially plant-available Zn- and Fe further affects yield as well as grain Zn and Fe concentrations [1]. Studies show that about half of cereal-cultivating soils globally are deficient in plant-available Zn [2], particularly acidic soils, and those in high-rainfall areas of the tropics. In spite of high total concentrations of Fe in tropical soils, high-level oxidation and fixation significantly affect their plant availability [3]. Deficiencies of Fe and Zn in soil results in reduced crop yield as well as quality since both minerals play major biological roles in plants, such as maintaining proper metabolic and physiological cellular processes [3].

Crop genotypes' breeding for resistance to Zn and Fe deficiency is a realistic and long-term solution to overcome problems related to Zn and Fe deficiency in soils [4]. However, breeding genotypes can require substantial time [5] as well as a relatively higher investment as compared to agronomic biofortification [6].

Fertilization with Zn and Fe is a common practice to help to combat Zn and Fe deficiencies as a short-term strategy [3,7]. Crop Zn and Fe deficiencies are most frequently amended by agronomic biofortification through soil application of Zn and Fe fertilizers [8]. Zinc sulphate ($ZnSO_4$) and ferrous sulphate ($FeSO_4$) are used extensively as sources of Zn and Fe fertilizers, because of their higher solubility in water and existence in both crystalline and granular forms [9]. Many studies have reported that the agronomic biofortification with Fe and Zn positively enhances crop yield and/or grain Fe and Zn concentrations in crops like wheat, maize and rice [1,2,3].

Crop selection in agronomic biofortification plays a critical role in its effectiveness. The identification and improvement of traditional or native crops that are highly adaptive to local climates and that can efficiently withstand biotic and abiotic stresses is crucial. Finger millet (*Eleusine coracana* L.) represents one of the critical plant genetic resources for the agriculture and food security of populations inhabiting arid, infertile and marginal lands [10]. In the semiarid tropics of eastern Africa, it is the major staple food for millions of resource-poor people and plays an important nutritional and economic role [11]. Finger millet is adaptable to adverse agro-ecological conditions with minimal inputs (fertilizer, pesticides and herbicides), showing low moisture stress and disease tolerance, is productive on marginal land where other crops cannot perform, and shows tolerance to acidic soil [12,13]. In Ethiopia, finger millet is the sixth most important crop after tef, wheat, maize, sorghum and barley. It has been produced on 480,343 hectares of land, from which ~1.2 M tons have been obtained at the national level per year [14]. Nationwide, ~1.55 M households are directly engaged in finger millet production, and the production has increased by 300% in the previous 20 years [14].

Previous reports on the impact of agronomic biofortification with Zn and Fe, regarding genotype as well as slope position, of indigenous crops like finger millet and teff in tropical smallholding farming systems are lacking. However, a report from Ethiopia has indicated that wheat yield was more strongly influenced by slope positions than either the nutrient sources or rates; thus, site-specific fertilizer treatment is strongly recommended [15]. Therefore, this paper reports on the effect of basal application of Zn and Fe fertilizer on grain yield and the yield attributes of three finger millet genotypes at different locations and slope positions.

3.2 Materials and Method

3.2.1. Field Experiment

The agronomic biofortification trials with Zn and Fe micronutrients were carried out at the Gojjam (11°41′54″ N 37°29′79″ E foot slope and 11°40′23″ N 37°30′29″ E hill slope) and Arsi Negelle (7°19′38″ N 38°38′54″ E foot slope and 7°18′43″ N 38°39′57″ E hill slope) areas on farmers' land (Figure 3.1). According to the classification of the agro-ecological zonation of Ethiopia, both sites are characterized as sub-humid midlands located between 1500–2300 m.a.s.l. and as having unimodal rainfall, with an average annual rainfall of 800–1200 mm [16]. The average temperature was recorded as 13 °C and 10 °C for the minimum and 23 °C and 26 °C for the maximum for Arsi Negelle and Gojjam, respectively, during the experimental seasons. The average temperature was 17.5 °C [17] and 20 °C [18] for Arsi Negelle and Gojjam, respectively. The experiment was laid out in a randomized complete block design (RCBD) (Supplementary Materials) with a factorial concept with 4 replications consisting of 15 treatment combinations involving 3 finger millet genotypes (Dagi-01, black in colour; Urji, white in colour and Meba, brown in colour) and 5 levels of fertilizer application (Table 3.1).

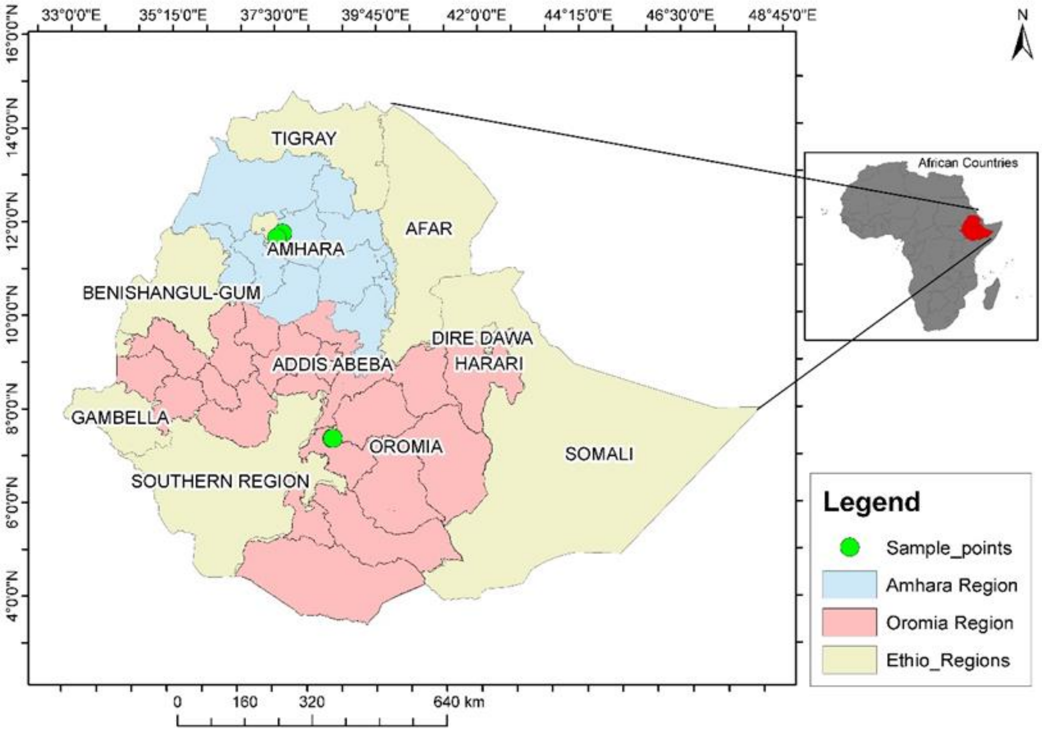

Figure 3.1. Map showing the study sites locations in Amhara and Oromia regions of Ethiopia

Table 3.1. Elemental application of nutrients in kg per hectare

Treatments	Zn	Fe	N	P	S	K
T1	5.5	4	32.1	3.59	15.89	31.2
T2	5.5	-	32.1	3.59	7.64	31.2
T3	-	-	9.63	1.1	1.57	9.36
T4	-	-	32.1	3.59	5.24	31.2
T5	-	4	32.1	3.59	13.49	31.2

T1: 25 kg $ZnSO_4 7H_2O$, 20 kg $FeSO_4 7H_2O$, 131 kg NPS, 60 kg K, and 54 kg urea ha^{-1}; T2: 25 kg $ZnSO_4 7H_2O$, 131 kg NPS, 60 kg K, and 54 kg urea ha^{-1}; T3: 131 kg NPS, 60 kg K, and 54 kg urea ha^{-1}; T4: 30% of T3; T5: 20 kg $FeSO_4 7H_2O$, 131 kg NPS, 60 kg K, and 54 kg urea ha^{-1}

3.2.2. Agronomic Management

The plot size was 4 m × 4 m, with gangways between plots being 1 m wide while the distances between the blocks and the borders were 0.5 m each. The experiment was repeated for two seasons, but only at Arsi Negelle (due to COVID-19 pandemic travel restrictions), and different farms were used in each year, sowed between mid-June and mid-July and harvested in November. Planting was done by hand drilling at a seed rate of 7 kg ha^{-1}. Each experimental plot had ten rows with 40 cm of inter-row spacing. NPKS, $ZnSO_4 7H_2O$ and $FeSO_4 7H_2O$ were applied at planting and urea was applied after 45 days at first weeding. All plots were weeded at least six times using human labour and no pesticides or herbicides were applied. Plant samples for data collection were tagged right after 100% crop germination and the crop was developed in a rainfed fashion, without irrigation.

3.2.3. Soil Sample Collection

The soil classes of both experimental locations, including the slopes, were mostly Nitisols [17, 18]. Soil samples were taken from a 60 m^2 circular plot in the experimental field. Five sub-sample sites were located; the first was at the centre. Two sub-sample points were selected at locations on a line through the plot centre along the crop rows, and two on a line orthogonal to the first through the plot centre. The ‘long’ axis of the sample array (with sample locations at 5.64 and 4.89 m) was oriented in the direction of crop rows, with the ‘short axis’ (with sample locations at 3.99 and 2.82 m) perpendicular to the crop rows. A single soil sub-sample was collected at each of the five sub-sample points with a Dutch auger with a flight of length 150 mm and diameter of 50 mm. Any plant material adhering to the auger was carefully removed, and the five sub-samples were stored in a single bag [19].

3.2.4. Soil Mineral Analysis

Soil sample digestion was performed following aqua regia digestion for the extraction of trace elements method (ISO 11466) (ISO, 1995). Briefly, 3 mL of 36% HCl and 9 mL of 70% HNO_3 were added in a microwave digestion tube containing 0.5 g of a ground soil sample. The digestion vessels were then transferred to the cavity of the microwave unit and the digestion was started at 185 °C and continued for 30 min. The digested sample supernatant was filtered carefully and diluted to 50 mL in a volumetric flask. CRM Wageningen-WEPAL ISE-850 (Calcareous soil) was used as a certified laboratory reference material and % mineral recovery was calculated. Blanks were also analysed at the same time. A three-step sequential extraction scheme for the fractionation of sulphur (S) was followed, using 0.01 M KNO_3, 0.016 M KH_2PO_4 and 10% tetra methyl ammonium hydroxide (TMAH) to determine soluble, exchangeable and organically-bound S fractions, respectively. The detailed procedure for soil sample collection, mineral analysis and three-step sequential extraction is reported elsewhere [19]. The soil mineral concentrations of each experimental site are presented in Table 3.2. The total calcium, potassium, boron, sulphur and iron contents of the soil samples were significantly different among the two locations and slope positions. The recovery for all minerals is between the acceptable ranges (85–120%).

Table 3.2. Mineral concentrations (mg/kg) of soil from the finger millet agronomic biofortification experimental sites in Ethiopia

Location	Slope	B	Mg	P	S	K	Ca	Fe	Zn
Arsi-Negelle	Foot	3.9±0.79^{A}	2052±47	2061±49	122.7±0.4	3227±185^{A}	4662±481^{A}	26918±1149^{A}	89±7
	Hill	3.0±0.70^{B}	1728±240	1725±240	105.8±7.3	2729±448^{B}	4050±918^{B}	23952±1804^{B}	105±10
	Mean	3.4±0.95^{a}	1890±259^{a}	1893±264^{a}	114.3±10.3^{a}	2978±464^{a}	4356±870 ^{a}	25435± 2320^{a}	97±13^{a}
Gojjam	Foot	1.0±0.37^{C}	1731±131	1731±131	136.6±6^{C}	934±33^{C}	1185±149^{C}	107973±3372^{C}	81±4
	Hill	0.1±0.08^{D}	1597±167	1597±167	207.1±42.8^{D}	859±85^{D}	1668±430^{D}	124304±5913^{D}	104±11
	Mean	0.55±0.5^{b}	1664±180^{a}	1664±180^{a}	171.8±47.3^{b}	897±82^{b}	1426±440^{b}	116138±10383^{b}	92±16^{a}

Note: Results labelled with different small letters are significantly different for location and those with different capital letter are significantly different for slope at the 0.05 probability level.

3.2.5. Agronomic Data Collection

The plant height, total tiller number, productive tiller number (number of basal tillers which bear mature ears), finger length of the longest spike and number of fingers per main ear were measured at the 50% maturity stage, which is after ~130 days after germination (International Board for Plant Genetic Resources [20]). Grain yield at 12% average moisture and biomass at 18% average moisture were taken (from the eight central rows) on a plot basis and then converted to a hectare basis. The plant height was measured from the ground to the tip of the inflorescence (ear). Finger length was measured from the base to the tip of the longest spike on the main tiller.

3.2.6. Data Analysis

Data collected for all agronomic quantitative characters were subjected to analysis of variance (ANOVA) using R software version 3.3.2 (R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing). The major descriptive statistics such as mean, range and standard deviation of each trait for the study genotypes, fertilizer levels, study location and slope positions were computed. Slope, fertilizer level and genotype were treated as fixed effects, whereas season, block within farm, farm within location and location were treated as random effects. Yield and biomass data were transformed to natural logarithms, as the dispersion of the random effects on the original scale appeared to increase with the fitted value. A comparison of means was done using Tukey's post hoc test ($p < 0.001$, 0.01, 0.05 and 0.1).

3.3 Result

Yield and Biomass Effects

Finger millet yield and biomass per hectare for each fertilizer treatment, genotype, location and slope position are presented in Figure 3.2, Figure 3.3, Figure 3.4 and Figure 3.5. The total and productive tiller numbers, number of fingers per main ear, plant height and finger lengths (cm) for each fertilizer treatment, genotype, location and slope are presented in Table 3.3 and Table 3.4. Yield and biomass showed a wide variation, ranging from 94 to 3828 kg and from 6.25 to 242.97 quintals per hectare, respectively. The maximum yield of the NPKS at the recommended rate in the current experiment (3594 kg ha^{-1}) was much higher than the national average [14] of 2504 kg. The finger number per main ear, total tiller number and productive tiller number ranged between 1 and 14, 1 and 16 and 1 and 14, respectively. The plant height and finger length ranged between 4 and 120 and 3 and 17 cm, respectively.

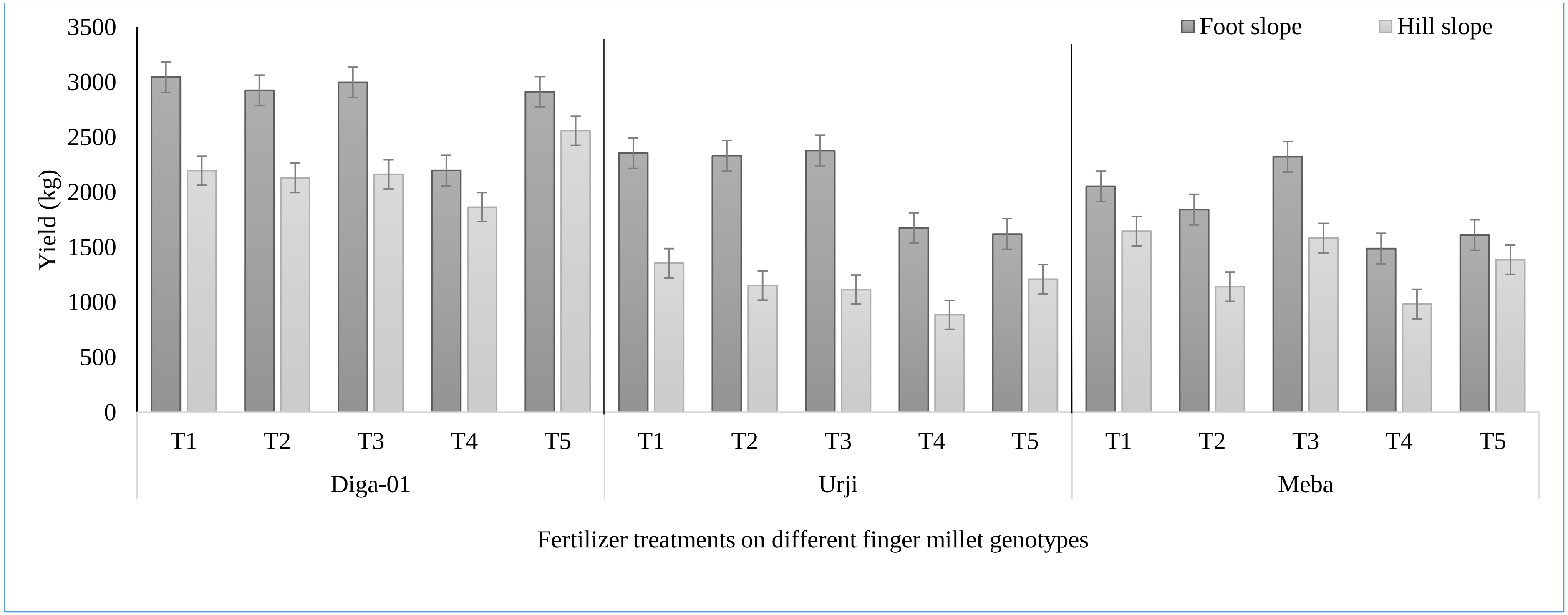

Figure 3.2. Effect of fertilizer treatment on yield (kg) of finger millet genotypes at Arsi Negelle as affected by slope position

T1: 25 kg $ZnSO_47H_2O$, 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T2: 25 kg $ZnSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T3: 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T4: 30% of T3; T5: 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}.

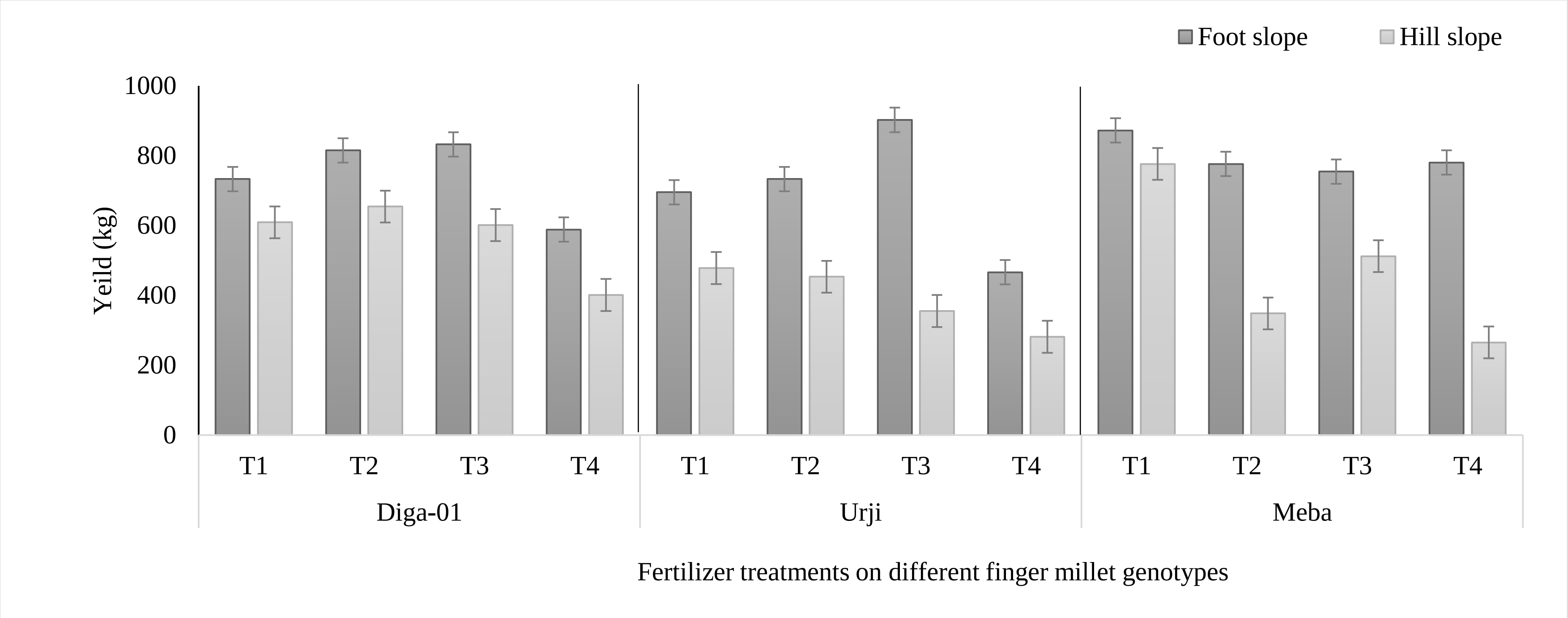

Figure 3.3. Effect of fertilizer treatment on yield (kg) of finger millet genotypes at Gojjam as affected by slope position

T1: 25 kg $ZnSO_47H_2O$, 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T2: 25 kg $ZnSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T3: 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T4: 30% of T3.

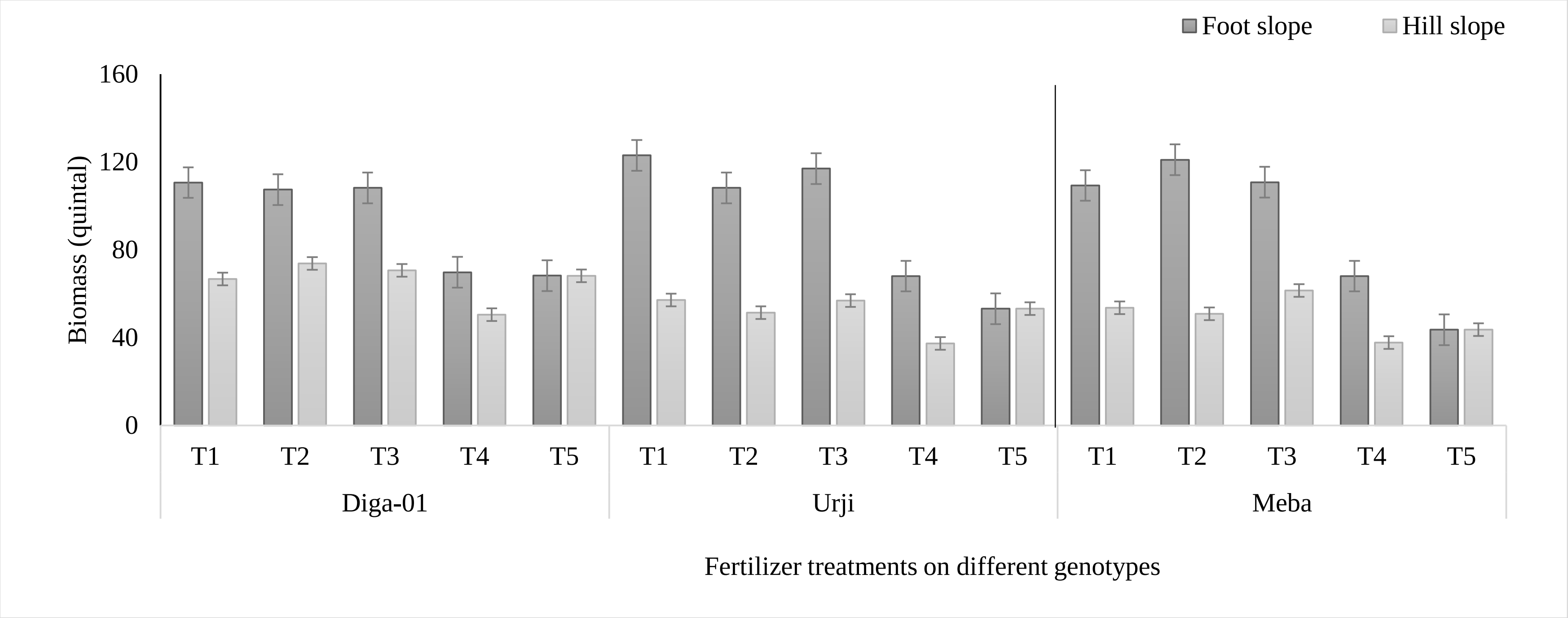

Figure 3.4. Effect of fertilizer on biomass (quintal) of finger millet genotypes at Arsi Negelle as affected by slope position

T1: 25 kg $ZnSO_47H_2O$, 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T2: 25 kg $ZnSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T3: 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T4: 30% of T3; T5: 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}.

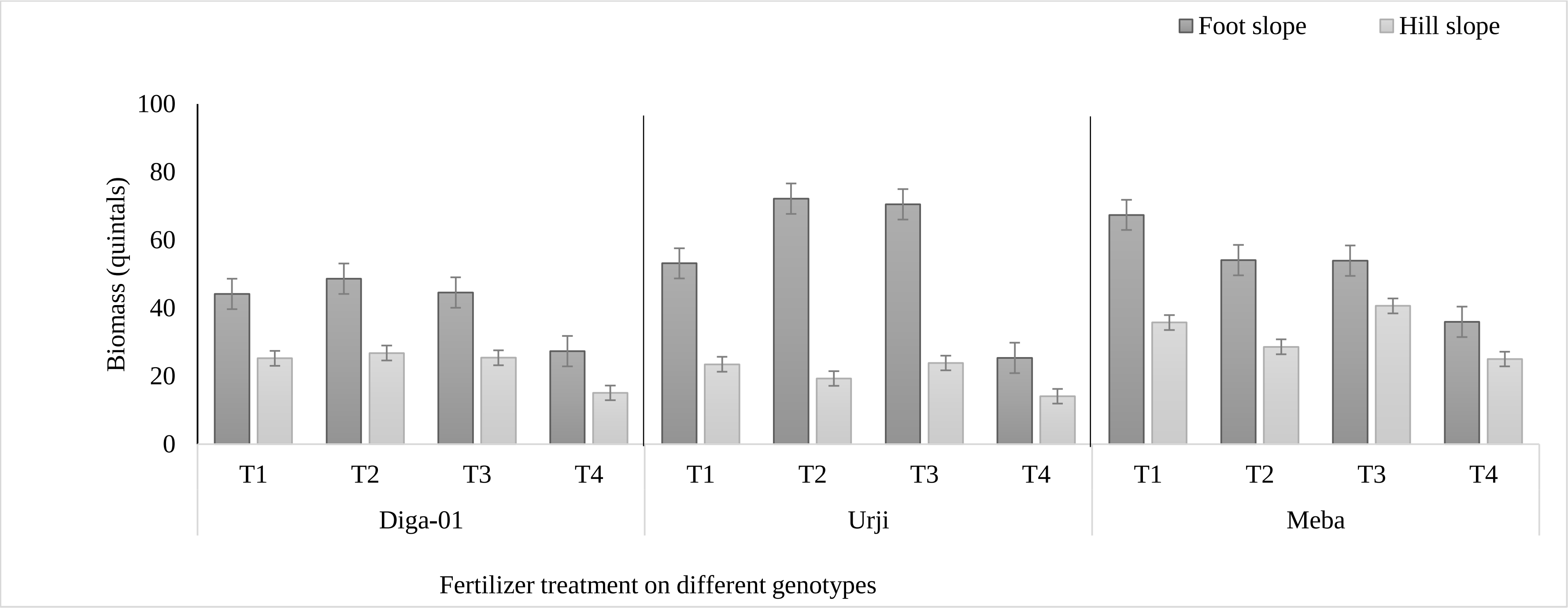

Figure 3.5. Biomass (quintal) of finger millet genotypes at Gojjam as affected by zinc and iron fertilization

T1: 25 kg $ZnSO_47H_2O$, 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T2: 25 kg $ZnSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T3: 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T4: 30% of T3.

Table 3.3. Effect of fertilizer treatment on yield traits of finger millet genotypes at Arsi Negelle as affected by slope postition

	Foot slope						Hill slope				
Variety	Fertilizer	Total tiller number	Productive tiller number	Finger no/ main ear	Plant Height (cm)	Finger Length (cm)	Total tiller number	Productive tiller number	Finger no/ main ear	Plant Height (cm)	Finger Length (cm)
Diga-01	T1	4.58±1.88	4.4±1.9	6.0±1.4	68.6±14.2	8.0±1.6	4.8±1.7	4.5±1.7	5.8±1.8	54.3±8.8	7.3±1.3
	T2	4.54±1.49	4.1±1.3	6.5±2	71.8±21.6	7.6±1.5	5.1±1.5	4.7±1.7	5.8±1.4	56.9±7.6	7.4±1.3
	T3	4.46±1.72	4.2±1.6	6.4±1.7	70.8±16.2	8.1±1.7	5.0±1.9	4.7±1.7	5.4±1.4	57.2±7.7	7.3±1.3
	T4	5.63±2.22	5.4±2.2	6.3±1.6	57.2±12	7.6±1.3	4.5±1.4	4.2±1.2	5.2±1.1	49.9±7.3	7.6±1.3
	T5	5.5±1.9	5.0±1.6	6.0±2.4	58.1±8.8	8.0±1.5	5.2±2	4.8±1.9	4.4±0.8	54.4±7.6	7.2±1.4
Urji	T1	4.38±1.58	3.9±1.6	7.1±2.2	64.6±16.4	7.7±1.4	4.9±1.9	4.1±1.5	7.3±1.8	52.1±7.2	7.9±1
	T2	4.86±1.74	4.3±1.5	7.9±1.8	65.6±18.2	8.3±1.5	4.9±2.3	4.3±1.7	7.4±1.8	51.8±8.3	7.6±1.4
	T3	4.81±1.96	4.3±2	7.6±1.6	64.8±13.4	7.9±1.7	5.4±2	4.5±1.4	7.2±1.9	53.0±8.3	7.4±1.3
	T4	5.6±2.9	5.2±2.8	7.8±1.5	56.2±13.7	8.3±1.7	5.6±2.6	4.9±2.5	7.0±1.6	47.1±6.8	6.6±1.2
	T5	5.0±2.2	4.1±1.9	6.7±1.3	49.0±9.7	7.5±2	5.4±3	4±1.8	6.8±1.8	48.4±7.9	6.8±1.7
Meba	T1	4.94±2.2	4.1±1.6	5.8±1.3	63.5±12.8	5.8±1.1	5.3±2	4.5±1.6	5.7±1.7	56.0±7.3	5.4±1.1
	T2	5.35±2.8	4.5±1.6	6.1±1.6	65.7±11	6.2±1.3	5.2±1.9	4.3±1.6	5.6±1.2	53.6±9	5.7±0.9
	T3	5.06±1.66	4.5±1.5	6.2±1.1	64.0±17.1	5.8±1.1	5.2±1.9	4.1±1.6	5.6±1.2	53.3±8.6	5.6±1.1
	T4	5.0 ±2.2	4.7±2.1	6.0±1.4	56.8±12.7	5.7±1.1	5.9 ±2.4	4.6±1.9	5.9±1.4	47.0±8.9	5.2±0.8
	T5	4.7±1.4	4.0±1	5.6±1.3	50.4±6.9	5.3±0.8	7.2±2	5.8±2.1	5.9±1.2	44.8±12.5	5.4±0.9

Table 3.4. Effect of fertilizer treatment on yield traits of finger millet genotypes at Gojjam as affected by slope position

	Foot slope						Hill slope				
Variety	Fertilizer	Total tiller number	Productive tiller number	Finger no/ main ear	Plant Height (cm)	Finger Length (cm)	Total tiller number	Productive tiller number	Finger no/ main ear	Plant Height (cm)	Finger Length (cm)
Diga-01	T1	1.35±0.6	1.31±0.6	6.1±1.5	65.1±17.1	9.8±1.6	1.56±0.7	1.45±0.7	5.7±1	46.5±6	9.2±1.1
	T2	1.41±0.8	1.34±0.8	5.3±1.2	61.3±12.9	9.6±1.9	1.83±0.9	1.77±0.9	5.2±0.8	44.2±7	9.5±1.9
	T3	1.38±0.6	1.31±0.6	5.8±1.2	66.2±9.1	9.8±1.5	1.45±0.7	1.39±0.7	5.2±1.2	44.0±6.9	8.8±1.1
	T4	1.36±0.4	1.29±0.4	5.1±0.9	57.4±10.8	9.7±1.9	1.44±0.6	1.35±0.6	4.8±1	42.3±10.4	7.7±1.4
Urji	T1	1.29±0.5	1.22±0.5	7.3±2.1	66.0±15.2	9.9±2.1	1.77±0.8	1.67±0.8	7.4±1.1	48.4±7.2	9.9±1.7
	T2	1.38±0.6	1.27±0.6	8.1±1.8	77.4±19.1	10.4±1.7	2.2±1.8	1.98±1.8	7.2±0.9	49.9±5	10.1±1.4
	T3	1.41±0.8	1.35±0.8	7.6±1.7	70.5±13.5	10.0±1.9	1.56±0.8	1.45±0.8	6.6±1.7	47.5±8.1	9.5±1.1
	T4	1.34±0.5	1.28±0.5	6.1±1.9	52.4±18.2	9.0±1.8	1.9±1.2	1.85±1.2	6.2±1.8	42.4±9.6	8.8±1.3
Meba	T1	1.24±0.5	1.17±0.5	4.8±0.8	70.3±7.9	7.6±1.4	1.74±1	1.67±1	4.7±0.7	60.1±7.6	7.5±1.6
	T2	1.33±0.6	1.25±0.6	4.9±1	69.0±11.7	8.2±1.4	1.55±0.6	1.45±0.6	4.7±1.2	52.0±9.9	6.7±2.2
	T3	1.42±0.7	1.37±0.7	4.8±1	70.4±11.1	7.5±1.5	1.5±0.9	1.5±0.9	5.0±1.2	57.3±9.2	7.4±2
	T4	1.44±0.7	1.38±0.7	4.7±1.5	60.3±11	7.2±1.3	1.8±1.2	1.8±1.2	4.2±1.1	51.3±9.2	6.9±1.6

A significant response from the Meba genotype to the combined $FeSO_47H_2O$ and $ZnSO_47H_2O$ fertilizer application at the Gojjam hill slope was exhibited; the average yield was increased by 51.6% (Table 3.5). The Diga-01 genotype responded significantly to $FeSO_47H_2O$ fertilizers at the Arsi Negelle hill slope position; an 18.3% average yield enhancement was recorded. A significant response was observed from the Urji genotype to $ZnSO_47H_2O$ fertilizers at the Gojjam hill slope position; a 27.6% average yield increase was observed. Irrespective of locations, slope position and genotype, grain yield was enhanced by 20% due to the soil application of $FeSO_47H_2O$ fertilizer.

Table 3.5. Effect of fertilizer treatment, genotype and slope position on finger millet yield

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Slope position	0.787	0.787	1	3.005	9.0260	0.05734 #
Fertilizer	7.2168	1.8042	4	274.966	20.6909	6.382e-15 ***
Genotype	10.3013	5.1506	2	274.491	59.0686	< 2.2e-16 ***
Fertilizer:genotype	0.8588	0.1073	8	275.993	1.2311	0.2807

Significance codes: *** <0.001; # < 0.1

Strong evidence ($p < 0.001$) was exhibited for the fertilizer's effect on finger millet yield irrespective of location, slope and genotype (Table 3.5). Similarly, the genotype shows strong evidence ($p < 0.001$) of effect on yield over location, slope and fertilizer. Moderate evidence ($p < 0.05$) has been seen for a slope position effect on finger millet yield irrespective of location, fertilizer application and genotype (Table 3.5). However, there is no evidence for the effect on yield due to the interaction of fertilizer and genotype (Table 3.5).

The fertilizer shows strong evidence ($p < 0.001$) of effect on finger millet biomass irrespective of location, slope and genotype (Table 3.6). Some evidence ($p < 0.05$) has been seen for a slope position effect on biomass irrespective of location, fertilizer and genotype (Table 3.6). Similarly, a slight genotype effect ($p < 0.05$) on biomass irrespective of location, slope and fertilizer was observed. However, the interaction of fertilizer and genotype exhibited no effect on biomass (Table 3.6).

Table 3.6. Effect of fertilizer treatment, genotype and slope position on finger millet biomass

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Slope position	1.1258	1.1258	1	3.004	13.5885	0.03453 *
Fertilizer	13.8891	3.4723	4	274.516	41.9113	< 2e-16 ***
Genotype	0.5367	0.2684	2	274.036	3.2392	0.0407 *
Fertilizer:genotype	0.7399	0.0925	8	274.873	1.1163	0.3522

Significance codes: *** <0.001; *< 0.05

Genotypes differed significantly in both the yield ($p < 0.001$) and biomass ($p < 0.05$) of finger millet, showing the average result of Diga-01 > Meba > Urji. Similarly, fertilizer treatment significantly ($p < 0.001$) affected both the yield and biomass of finger millet, showing the average results of T5 > T1 > T3 > T2 > T4 and T3 > T1 > T2 > T5 >T4, respectively.

Strong evidence ($p < 0.001$) was seen for the fertilizer's effect on finger millet plant height as well as on finger length irrespective of location, slope and genotype. The genotype shows strong evidence ($p < 0.001$) for its effect on finger length and for some effect on plant height ($p < 0.05$). Slope position shows weak evidence ($p < 0.1$) for its effect on plant height.

3.4 Discussion

The present study reports finger millet genotypic responses to Zn and Fe agronomic biofortification, location and slope position in yield and biomass. Irrespective of genotype, locations and slope positions, grain yield was enhanced by 20% due to the soil application of $FeSO_4$ fertilizer. However, different finger millet genotypes responded differently to both fertilizer treatment and location with respect to yield and yield traits. This suggests that finger millet genotypes differ in their ability to remobilize and retranslocate deposited Zn and Fe, which plays a critical role in the improvement of the partitioning of carbohydrates from the leaves to the reproductive parts, affecting the yield and yield attributes. Therefore, the current finding should be taken into consideration in evaluating cereal genotypes for their responses to agronomic biofortification. To our knowledge, there is no available previous data on finger millet with Zn, Fe, or combined fertilizer, with genotype, location, and slope position effects, on grain yield and yield traits. Thus, this experiment is the first of its kind to report the triple impact of Zn and Fe agronomic biofortification, genotype, and environment (location and slope position) on the grain yield and biomass of finger millet. However, previous studies show that different finger millet genotypes responded differently to NPK fertilization in India [21], to phosphorus fertilizer in locations in Kenya [22], and to location in Ethiopia [23]. On the other hand, wheat and rice genotypes responded differently to Zn fertilization in Turkey [8] and to Zn fertilization as well as climate in India [24], respectively.

4.1. Finger Millet Genotypic Response to Zn Fertilizer towards Yield Affected by Location and Slope Position

The present study indicates that the Urji genotype responds significantly to $ZnSO_4$ fertilizers at the Gojjam hill slope position, where a 27.6% yield increase was observed. The possible reason behind the fact that finger millet responded well at the Gojjam hill slope position to Zn fertilization is that the soil sulphur concentration is significantly higher at the Gojjam hill slope position (Table 3.1), since sulphur is reported to enhance the solubility of Zn and its uptake by the plant [25]. On the other hand, the application of $ZnSO_4$ fertilizers significantly increased the total and productive tiller number for the Urji genotype at the hill slope position (Table 3.4), and this might have also played a major role in the yield increase. There are no available previous data that explore the impact of Zn fertilization on finger millet grain yield. However, previous research on wheat, rice, maize, sorghum, etc, has explored the effect of Zn fertilization on grain yield. A study from India showed a 14.2% yield increase as a result of the application of Zn fertilization [26]. Similarly, Phattarakul et al. [27] reported an increase of 10% in crop yield in their experiment, which was conducted in China and India. Another experiment from India indicated a 23.5% increase in grain yield by applying Zn fertilization [28]. An increase in yield of up to 33% was observed as a result of the application of Zn fertilizer [24]. Narwal et al. [29] also found a 5% yield enhancement by applying Zn fertilizer. Overwhelming evidence from all over the world indicates that the application of Zn fertilizer improves crop yields [1,2,8,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45]. The positive response of yields in cereal to Zn fertilization in both current and previous studies is possibly due to the enhancement of plants' available Zn for uptake [8], which in turn helps the plants achieve better protein breakdown and enzyme activation, resulting in higher vegetative growth and yield increases [28]. However, one study from Thailand and Turkey shows little or insignificant effect of Zn fertilizer application on yield [27]. The irresponsiveness of rice to Zn fertilization in a previous study was possibly due to the absence of sulphur fertilization, since sulphur has been reported to enhance the solubility of Zn and its uptake by spring wheat [25]. In addition to that, a lower soil Zn concentration was reported (ranging from 0.5 to 6.5 mg kg^{-1} in a previous study, in contrast to 85 to 105 mg kg^{-1} in the present study). The other possibility might be that different crop genotypes responded differently to Zn fertilizer treatment, climate and environment (location and slope position), as was witnessed in the current as well as previous studies [8,23,24,45].

4.2. Finger Millet Genotypic Response to Fe Fertilizer towards Yield Affected by Location and Slope Position

The present study indicates that the Diga-01 genotype responded significantly to $FeSO_4$ fertilizer application at the Arsi Negelle hill slope position, in which an 18.3% average yield enhancement was recorded. The possible reason behind the fact that finger millet responded well at the Arsi Negelle hill slope position to Fe fertilization is that the soil potassium concentration is significantly higher at the Arsi Negelle hill slope position (Table 3.1), since it is reported that potassium seems to have a very specific role in the plant for the maximum utilization of Fe [46,47]. On the other hand, application of $FeSO_4$ fertilizers significantly increased the total and productive tiller number for the Diga-01 genotype at the hill slope position (Table 3.3), and this might also play a major role in yield increase. Even though no data are available for Fe fertilization’s impact on finger millet grain yield, a few previous experiments on wheat, barley and oats have investigated the effect of Fe fertilization on grain yield and are discussed with the current result. The agronomic biofortification of Fe fertilizer is less well studied as compared to Zn fertilizer. For instance, a study from India indicated an enhancement of 13% in yield due to the application of Fe fertilizer [29]. The positive response of yield to Fe fertilization in both current and previous studies is possibly due to the enhancement of plant-available/soluble Fe for uptake [8], which in turn helps the plant achieve better chlorophyll synthesis, protein and carbohydrate metabolism and enzyme activation, resulting in better vegetative growth and yield increases [48]. However, studies from Turkey and Canada on Fe biofortification showed no yield improvement [30,49]. This might be due to three possible reasons: the first reason could be that, in general, different crops and genotypes responded differently to mineral fertilization as well as location [1,23,24]. The second possibility is that when applied to calcareous soils, Fe rapidly converted into unavailable forms, and the poor mobility of Fe in phloem makes the Fe fertilization impact limited or unsuccessful [2,44]. It is also possibly due to the graminaceous species’ release of phytosiderophores (Fe-mobilizing compounds) to solubilize and absorb Fe from calcareous soils with low Fe concentrations; thus, they can maintain adequate plant growth by satisfying Fe demand without the requirement for Fe fertilization [50,51].

3.5 Conclusions

Finger millet genotypes greatly influenced the response to agronomic biofortification of Zn and Fe fertilizer in the present study, which indicates the varied yield and yield traits performances of the genotypes across different environments (location and slope position). This reveals the vitality of experimenting on finger millet genotypes' responses to Zn and Fe fertilizer in different environments (location and slope positions) prior to a scale-up for mass production. The soil application of 20 kg of $FeSO_47H_2O$ per hectare along with the recommended rate of NPKS could be an excellent agronomic biofortification strategy to enhance the yields of all genotypes in the study areas and in areas with similar agro-ecologies. Moreover, the soil application of 20 kg $ZnSO_47H_2O$ and of a combined 20 kg $FeSO_47H_2O$ and 25 kg $ZnSO_47H_2O$ in Urji and Meba, respectively, at the Gojjam hill slope and in areas with similar agro-ecologies, could be a premium agronomic biofortification strategy to improve finger millet grain yield. Future studies as well as the development of programs on agronomic biofortification should consider environmental (location and slope position) effects in addition to the main fertilizer effect, which is a gap in the current knowledge.

Author Contributions

D.T., D.G., E.J.M.J., R.M.L., T.A. and M.R.B.: conceptualization. D.T.: data collection, supervision, and original draft preparation. D.T. and T.A.: experimental design. D.T. and E.H.B.: soil sample preparation and analysis. D.T., D.G. and R.M.L.: statistical analysis. D.T., D.G., E.J.M.J., R.M.L., E.H.B., L.W., T.A. and M.R.B.: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Supplementary material

Experimental design of the field treatment

Design: CRBD at slope position level (n= 3 genotypes x 5 level fertilizer application x 4 replications).

Slope class: Hill slope

Farmer 1

Diga-01 NPKS	**Meba** 30%NPKS	**Diga-01** NPKS+Zn	**Urji** NPKS	**Meba** NPKS
Urji NPKS+Zn+ Fe	**Diga-01** NPKS+Zn+ Fe	**Urji** 30%NPKS	**Meba** NPKS+Zn+ Fe	**Diga-01** NPKS+Fe
Meba NPKS+Zn	**Urji** NPKS+Fe	**Meba** NPKS+Fe	**Diga-01** 30%NPKS	**Urji** NPKS+Zn

Farmer 2

Meba NPKS+Zn+ Fe	**Diga-01** NPKS+Fe	**Meba** NPKS+Fe	**Diga-01** NPKS+Zn	**Urji** NPKS+Fe
Diga-01 NPKS	**Urji** NPKS	**Urji** 30%NPKS	**Meba** NPKS	**Diga-01** 30%NPKS
Urji NPKS+Zn	**Meba** 30%NPKS	**Diga-01** NPKS+Zn+ Fe	**Urji** NPKS+Zn+ Fe	**Meba** NPKS+Zn

Farmer 3

Urji NPKS+Zn	**Meba** 30%NPKS	**Diga-01** 30%NPKS	**Meba** NPKS+Zn+ Fe	**Diga-01** NPKS+Zn
Meba NPKS	**Urji** NPKS+Zn+ Fe	**Urji** NPKS	**Diga-01** NPKS	**Meba** NPKS+Fe
Diga-01 NPKS+Zn+ Fe	**Diga-01** NPKS+Fe	**Meba** NPKS+Zn	**Urji** NPKS+Fe	**Urji** 30%NPKS

Farmer 4

Diga-01 30%NPKS	**Urji** NPKS	**Urji** 30%NPKS	**Meba** NPKS+Fe	**Diga-01** NPKS
Diga-01 NPKS+Zn	**Meba** NPKS+Zn+ Fe	**Diga-01** NPKS+Zn+ Fe	**Urji** 30%NPKS	**Urji** NPKS+Fe
Meba 30%NPKS	**Urji** NPKS+Zn+ Fe	**Meba** NPKS+Zn	**Diga-01** NPKS+Fe	**Meba** NPKS

Slope class: Foot slope

Farmer 1

Diga-01 NPKS	**Meba** 30%NPKS	**Diga-01** NPKS+Zn	**Urji** NPKS	**Meba** NPKS
Urji NPKS+Zn+ Fe	**Diga-01** NPKS+Zn+ Fe	**Urji** 30%NPKS	**Meba** NPKS+Zn+ Fe	**Diga-01** NPKS+Fe
Meba NPKS+Zn	**Urji** NPKS+Fe	**Meba** NPKS+Fe	**Diga-01** 30%NPKS	**Urji** NPKS+Zn

Farmer 2

Meba NPKS+Zn+ Fe	**Diga-01** NPKS+Fe	**Meba** NPKS+Fe	**Diga-01** NPKS+Zn	**Urji** NPKS+Fe
Diga-01 NPKS	**Urji** NPKS	**Urji** 30%NPKS	**Meba** NPKS	**Diga-01** 30%NPKS
Urji NPKS+Zn	**Meba** 30%NPKS	**Diga-01** NPKS+Zn+ Fe	**Urji** NPKS+Zn+ Fe	**Meba** NPKS+Zn

Farmer 3

Urji NPKS+Zn	**Meba** 30%NPKS	**Diga-01** 30%NPKS	**Meba** NPKS+Zn+ Fe	**Diga-01** NPKS+Zn
Meba NPKS	**Urji** NPKS+Zn+ Fe	**Urji** NPKS	**Diga-01** NPKS	**Meba** NPKS+Fe
Diga-01 NPKS+Zn+ Fe	**Diga-01** NPKS+Fe	**Meba** NPKS+Zn	**Urji** NPKS+Fe	**Urji** 30%NPKS

Farmer 4

Diga-01 30%NPKS	**Urji** NPKS	**Urji** 30%NPKS	**Meba** NPKS+Fe	**Diga-01** NPKS
Diga-01 NPKS+Zn	**Meba** NPKS+Zn+ Fe	**Diga-01** NPKS+Zn+ Fe	**Urji** 30%NPKS	**Urji** NPKS+Fe
Meba 30%NPKS	**Urji** NPKS+Zn+ Fe	**Meba** NPKS+Zn	**Diga-01** NPKS+Fe	**Meba** NPKS

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Chapter 4: Impact of zinc and iron agronomic biofortification on grain mineral concentration of finger millet varieties as affected by location and slope

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Abstract

Background: Food crop micronutrient concentrations can be enhanced through agronomic biofortification, with the potential to reduce micronutrient deficiencies among rural population if they have access to fertilizers. Here we reported the impact of agronomic biofortification on finger millet grain zinc (Zn) and iron (Fe) concentration.

Methods: A field experiment was conducted in farmers' fields in Ethiopia in two locations; over two seasons in one district (2019 and 2020), and over a single season (2019) in a second district. The experimental design had 15 treatment combinations comprising 3 finger millet varieties and 5 soil-applied fertilizer treatments: (T1) 20 kg ha^{-1} $FeSO_4$ + 25 kg ha^{-1} $ZnSO_4$ + NPKS; (T2) 25 kg ha^{-1} $ZnSO_4$ + NPKS; (T3) NPKS; (T4) 30% NPKS; (T5) 20 kg ha^{-1} $FeSO_4$ + NPKS. The treatments were studied at two slope positions (foot and hill), replicated four times in a randomized complete block design.

Results: Grain Zn concentration increased by 20% in response to Fe and Zn and by 18.9% due to Zn addition. Similarly, grain Fe concentration increased by 21.4% in T1 and 17.8% in T5 (Fe). Zinc fertilizer application ($p < 0.001$), finger millet variety ($p < 0.001$), and an interaction of Fe and Zn had significant effect on grain Zn concentration. Iron fertilizer ($p < 0.001$) and interactive effect of Fe fertilizer and finger millet variety ($p < 0.01$) had significant effects on grain Fe concentration. Location but not slope position was a source of variation for both grain Zn and Fe concentrations.

Conclusion: Soil application of Zn and Fe could be a viable strategy to enhance grain Zn and Fe concentration to finger millet grain. If increased grain Zn and Fe is bioavailable, it could help to combat micronutrient deficiencies.

4.1 Introduction

Micronutrient deficiencies (MNDs), which affect more than 2 billion people globally (1), is predominantly a result of intake of monotonous diets dominated by foods of low nutrient content (2). MNDs are more prevalent in developing countries where the diet is dominated by staple crop-based foods (3). In Ethiopia, a high prevalence of Zn (72%) and Fe (34.4%) deficiencies has been reported based on biomarkers of status (4). The nutritional quality including Zn and Fe content of cereals (5) also varies geospatially in Ethiopia. Zinc and Fe deficiencies can lead to impaired physical growth and cognitive functions, reduced resistance to infections, metabolic disorders, and increased prenatal morbidity (6).

MNDs can be addressed through different program that are either targeted on enhancing vitamin and mineral intake, or on reducing loss of nutrients from the body. Strategies can include dietary diversification, food fortification, and supplementation. In addition, public health measures such as deworming, vaccination, improved water, sanitation and hygienic practices, improved health care system and nutrition education are crucial interventions to prevent MNDs (7). Each strategy has strengths and limitations, and they should be considered in the context of local conditions. For example, dietary diversification is the most sustainable and preferred strategy since it can potentially address the root cause of MNDs. However, availability and affordability of the diversified foods can be barriers in resource poor societies. Food fortification can potentially have wider impact and be more cost-effective compared to supplementation. However, food fortification is limited to the centrally processed foods and thus difficult to address societies that are dependent on local food sources. Supplementation is preferred when the MND is severe and there is a need to provide the quickest improvements, although can present many logistical challenges (8).

Agronomic biofortification is a strategy to increase micronutrient content in the edible part of food crops through the application of mineral fertilizers (9). This approach can enrich food crops with multiple elements at a time and can reach resource poor rural populations, providing they have access to fertilizers. On the other hand, repeated and excess use of mineral fertilizers may cause soil and water contamination over time suggesting the need for regular monitoring of the environmental impact of agronomic biofortification (10). For agronomic biofortification to be effective, targeting food crops and varieties known to well adapt in their local environment is important (11).

Finger millet has several agronomic merits that can make it well-adapted to certain environments, including tolerance to moisture stress and soil acidity as well as resistance to disease (12). Finger millet also has appreciable nutritional content, e.g., high calcium (Ca) concentration 450 mg 100 g^{-1} (10-fold greater than milk on a volume basis) (5, 13). It is also a good source of other micronutrients (5) and protein (15.58%) (14). In addition, finger millet grain is gluten free, which makes it more attractive than wheat to some consumers (15). Finger millet is an indigenous crop to Ethiopia, and the sixth most important cereal crop after teff, wheat, maize, sorghum, and barley. It is produced on ~480,000 hectare of land, yielding about 1.2 M tonnes *per annum* (16). To our knowledge, there is no available previous data on finger millet for the effect of Zn, Fe, or combined fertilizers on grain Zn and Fe concentration. Therefore, this study aims to determine the potential impact of agronomic biofortification on finger millet grain Zn and Fe concentration. In addition, this study aims to investigate influence of varietal and environmental factors on Zn and Fe biofortification response. For example, it has been observed that the amount of plant available Zn can vary in different landscape positions of Ethiopian mixed cereal cropping systems (17).

4.2 Materials and methods

4.2.1 Field experiment

Agronomic biofortification experiments with Zn and Fe fertilizers were carried out in farmer fields, in two districts (locally known as *Woreda*) in the Amhara and Oromia regions, Ethiopia: Gojjam (11°41′54”N 37°29′79″E foot slope and 11°40′23”N 37°30′29″E hill slope) and Arsi Negelle (7°19′38”N 38°38′54″E foot slope and 7°18′43”N 38°39′57″E hill slope), respectively. According to the agro-ecological classification of Ethiopia, both sites are characterized as sub-humid midlands located between 1,500 and 2,300 m.a.s.l. and receive an average annual rainfall of 800–1,200 mm (18). The experiment consisted of 15 treatment combinations: 3 finger millet varieties (Diga-01, black grain colour; Urji white grain colour; Meba, brown grain colour) and 5 soil-applied fertilizer treatments as indicated below:

T1: 25 kg ha^{-1} $ZnSO_47H_2O$, 20 kg ha^{-1} $FeSO_47H_2O$, 131 kg ha^{-1} NPS, 60 kg ha^{-1} potassium (K), 54 kg ha^{-1} urea.

T2: 25 kg ha^{-1} $ZnSO_47H_2O$, 131 kg ha^{-1} NPS, 60 kg ha^{-1} K, 54 kg ha^{-1} urea.

T3: 131 kg ha^{-1} NPS, 60 kg ha^{-1} K, 54 kg ha^{-1} urea (control).

T4: 30% rate of T3 (Negative control).

T5: 20 kg ha^{-1} $FeSO_47H_2O$, 131 kg ha^{-1} NPS, 60 kg ha^{-1} K, 54 kg ha^{-1} urea.

Note: The negative control was included to observe the dilution effect. This is because reports show that considerable number of farmers reported to use very low amount of fertilizer as compared to the recommendation (19).

A randomized completed block design (RCBD) was used with 4 replications. The plot size was 4 m x 4 m, with gangways of 1 m width between plots while the distance between the block was 0.5 m. The experiment was repeated for two seasons but only at Arsi Negelle (due to Covid-19 pandemic travel restriction in Gojjam). Different farms were used in each year, sowed between mid-June and mid-July. Harvesting of the trial crop was on the first week of November and end November at Arsi Negelle and Gojjam, respectively.

4.2.2 Sample collection

Soil samples for mineral analysis were collected from a 60 m^2 circular plot in each of the experimental fields from both locations. Five sub-sample sites were located, the first at the centre, then two sub-sample points were selected at locations on a line through the plot centre along the crop rows, and two on a line orthogonal to the first through the plot centre. The ‘long’ axis of the sample array (with sample locations at 5.64 m and 4.89 m) was oriented in the direction of crop rows with the ‘short axis’ (with sample locations at 3.99 m and 2.82 m) perpendicular to the crop rows. A single soil sample was collected (to 20 cm depth) at each of the five sub-sample points with a Dutch auger with a flight of length 150 mm and diameter 50 mm. Any plant material adhering to the auger was carefully removed, and the sub-samples were aggregated and stored in a single bag. The detail procedure on soil sample collection, processing and mineral analysis follows Gashu et al. (20).

Matured and dried finger millet crop fingers were taken from each plot and the crop samples were hand threshed to produce approximately 1 kg of grain representing a sample and whole-grain samples were packed in sample envelope (5).

4.2.3 Sample preparation

Whole-grain samples were air-dried in sample bags. The samples were ground in a domestic stainless-steel coffee grinder, which was wiped clean before use and after each sample with a non-abrasive cloth. All preparations were done away from sources of soil and dust contamination. A 20 g subsample (following a representative quartering system) of the ground finger millet was then shipped to the University of Nottingham, UK. Soil samples were oven-dried at 40°C for 24–48 h depending on the moisture content of the soil. Preparation took place in a soil laboratory to avoid cross-contamination. Plant material and stones were removed from soil samples, which was then disaggregated and sieved to pass through 2 mm (5). This material was representatively quartered to produce subsamples. A 150 g subsample of soil was poured into a self-seal bag, labelled and shipped to the UK for analysis in the laboratories at the University of Nottingham.

4.2.4 Soil and grain mineral analysis

The soil samples were digested using Aqua-Regia for mineral analysis (20). A certified reference material (CRM Wageningen, WEPAL ISE-850, Calcareous Soil) was digested and analysed for mineral concentration for quality control purpose of the method. Operational blanks were also analysed at the same time following the same procedure to determine limits of detection (LODs). A three-step sequential extraction scheme for the fractionation of sulfur (S) was followed, using 0.01 M KNO_3, 0.016 M KH_2PO_4, and 10% tetra methyl ammonium hydroxide (TMAH) to determine soluble, exchangeable, and organically bound S fractions, respectively. The detailed procedure for soil mineral analysis and three-step sequential extraction for S is reported elsewhere (20–22).

Hot plate acid digestion was employed for grain sample digestion using methods described in Kumssa et al. (22). Briefly, 0.2 g of finger millet grain samples were weighed in digestion tubes and placed into heating blocks (Multicube 48, Anton Paar Ltd., UK). Then, 8 mL of concentrated HNO_3 (trace metal grade, Fisher Chemical, United States) was added to each tube and left for 30 min at room temperature. The samples were heated for 2 h at 115°C, left to cool for 10 min, and the samples were diluted to 50 mL using milliQ water (18.2 MΩ cm; Fisher Scientific). A 1 mL aliquot was transferred into an inductively coupled plasma tube and diluted again to 10 mL using milliQ water. A certified reference material (CRM Wheat 1567b, National Institute of Standards and Technology, Gaithersburg, MD, United States) was used to determine % recovery. Operational blanks were also analysed to determine limits of detection (LODs). Soil and crop sample mineral concentrations were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Scientific, Germany).

4.2.5 Statistical analysis

Data were analysed using R software version 3.3.2. The data were presented as mean ± standard deviation (SD). The data were analysed with a linear mixed model to compare mineral concentrations among control and fortified grain samples. Slope, fertilizer, and variety were treated as fixed effects whereas season, block within farm, farm within location, and location were treated as random effects. For logistical reasons treatments and varieties were randomized within farms, and farms were selected from slope positions within locations. The replication for slope position is thus very limited, and the model is prone to singularities. For several variables, slope was therefore dropped as a fixed effect and treated as a random effect for grain Fe concentration data. Then, variance component for the slope random effect was checked to determine how important slope might be relative to other factors. Skewness of the residuals for grain Zn and Fe concentration data and histograms were examined to decide whether any transformation was required before proceeding to interpretation of the outputs (see Supplementary material).

The fixed effect for treatments can be partitioned into four orthogonal contrasts, selected prior to data analysis, to test specific hypotheses. These were as follows.

C1: The comparison between the mean grain Zn for the 0.3NPKS treatment and all the treatments with NPKS at recommended rate.

C2: Within the full NPKS rate, the Fe main effect (difference between treatments with Fe and no Fe).

C3: Within the full NPKS rate, the Zn main effect (difference between treatments with Zn and no Zn).

C4: The Fe/Zn interaction: does the response to Zn depend on the level of Fe and vice versa.

4.3 Results

Mineral concentrations of soil samples from both trial locations at each slope position is presented in Table 4.1. Calcium, potassium, boron, sulphur, and iron content of soil samples were significantly different among the two locations and slope positions. The recovery for all minerals is between the acceptable ranges (85–120%). Fe and Zn agronomic biofortification response of finger millet samples for each fertilizer treatment at different location and slope position is indicated in Figures 4.1, 4.2. The recovery percentage for grain Fe and Zn concentration was 91.0 and 96.4%, respectively.

Grain Zn concentration was significantly different ($p < 0.001$) between fertilizer treatments (see Supplementary materials). There was 20 and 18.9% increase in grain Zn concentration in response to application of combined Fe and Zn, and Zn-only fertilizers, respectively (Figures 4.1, 4.2). There was no significant difference in grain Zn concentration between 30% NPKS and NPKS at recommended rate. Irrespective of fertilizer treatment, location and slope position, finger millet varieties differed significantly ($p < 0.001$) with respect to Zn concentration, showing the average result of Diga-01 < Meba < Urji (Figure 4.2). There is no evidence for an interaction between fertilizer and variety effect on grain Zn concentration (see Supplementary material).

Table 4.1. Mineral concentrations (mg/kg) of soil from the finger millet agronomic biofortification experimental sites in Ethiopia

Location	Slope	B	Mg	P	S	K	Ca	Fe	Zn
Arsi-Negelle	Foot	3.9±0.79^{A}	2052±47	2061±49	122.7±0.4	3227±185^{A}	4662±481^{A}	26918±1149^{A}	89±7
	Hill	3.0±0.70^{B}	1728±240	1725±240	105.8±7.3	2729±448^{B}	4050±918^{B}	23952±1804^{B}	105±10
	Mean	3.4±0.95^{a}	1890±259^{a}	1893±264^{a}	114.3±10.3^{a}	2978±464^{a}	4356±870 ^{a}	25435± 2320^{a}	97±13^{a}
Gojjam	Foot	1.0±0.37^{C}	1731±131	1731±131	136.6±6^{C}	934±33^{C}	1185±149^{C}	107973±3372^{C}	81±4
	Hill	0.1±0.08^{D}	1597±167	1597±167	207.1±42.8^{D}	859±85^{D}	1668±430^{D}	124304±5913^{D}	104±11
	Mean	0.55±0.5^{b}	1664±180^{a}	1664±180^{a}	171.8±47.3^{b}	897±82^{b}	1426±440^{b}	116138±10383^{b}	92±16^{a}

Note: Results labelled with different small letters are significantly different for location and those with different capital letter are significantly different for slope at the 0.05 probability level.

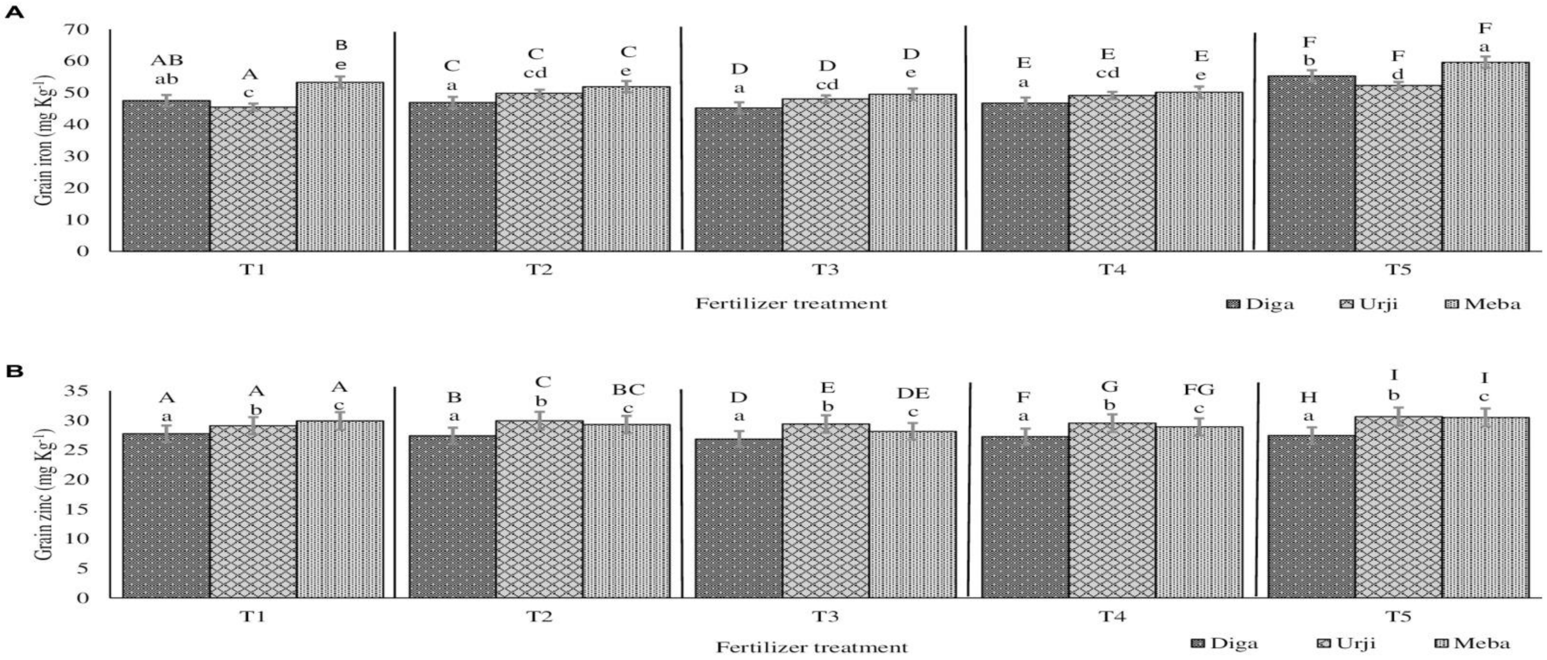

FIGURE 4.1: Grain iron **(A)** and zinc **(B)** concentration of finger millet at Arsi Negelle as affected by genotype and zinc and iron fertilization. T1: 25 kg ZnSO47H2O, 20 kg FeSO47H2O, 131 kg NPS, 60 kg K, 54 kg urea ha−1; T2: 25 kg ZnSO47H2O, 131 kg NPS, 60 kg K, 54 kg urea ha−1; T3: 131 kg NPS, 60 kg K, 54 kg urea ha−1; T4: 30% of T3; T5: 20 kg FeSO47H2O, 131 kg NPS, 60 kg K, 54 kg urea ha−1. Columns designated by different lowercases (a, b, c, d, e) have significantly different response to fertilizer treatment for single genotype. Columns designated by different uppercases (A, B, C, D, E, F, G, H, I) have significantly different response to genotypes for single fertilizer treatment.

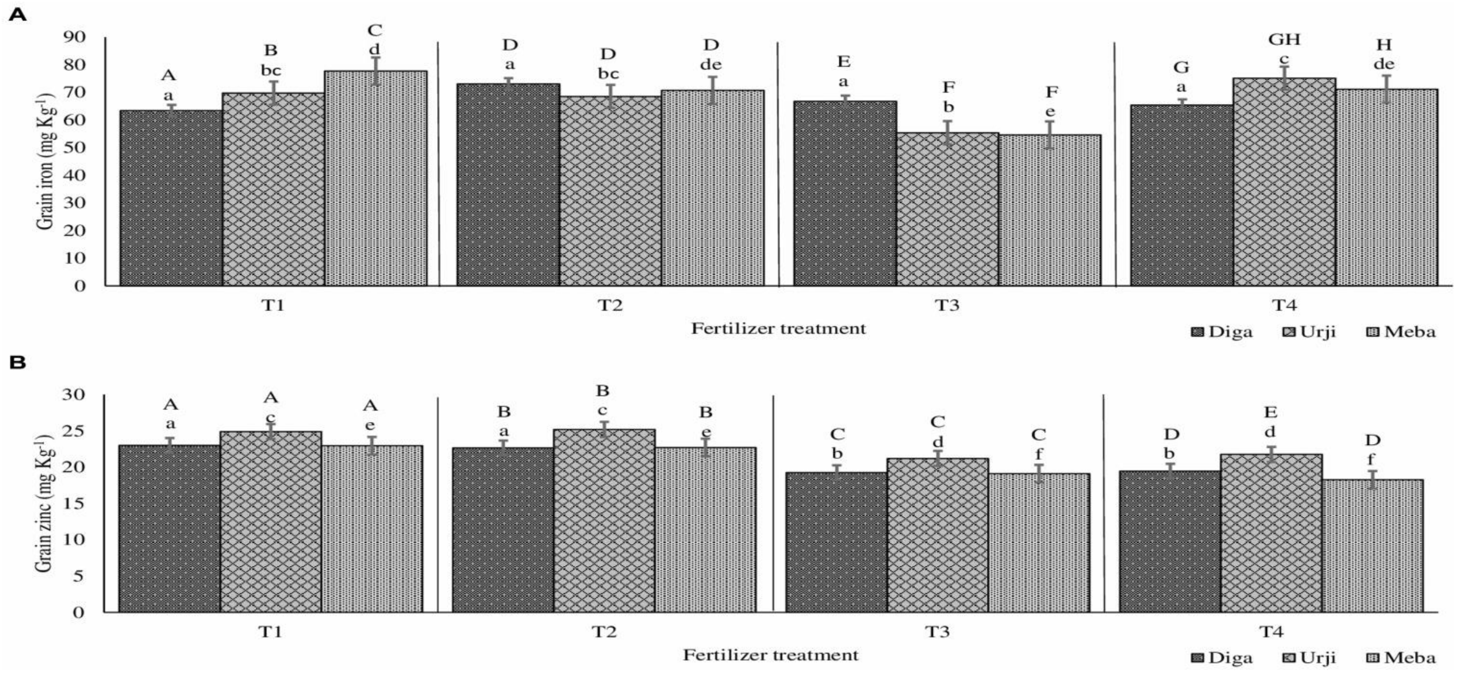

FIGURE 4.2: Grain iron **(A)** and zinc **(B)** concentration of finger millet at Gojjam as affected by genotype and zinc and iron fertilization
T1: 25 kg ZnSO47H2O, 20 kg FeSO47H2O, 131 kg NPS, 60 kg K, 54 kg urea ha−1; T2: 25 kg ZnSO47H2O, 131 kg NPS, 60 kg K, 54 kg urea ha−1; T3: 131 kg NPS, 60 kg K, 54 kg urea ha−1; T4: 30% of T3; T5: 20 kg FeSO47H2O, 131 kg NPS, 60 kg K, 54 kg urea ha−1. Columns designated by different lowercases (a, b, c, d, e, f) have significantly different response to fertilizer treatment for single genotype. Columns designated by different uppercases (A, B, C, D, E, F, G, H, I) have significantly different response to genotypes for single fertilizer treatment.

The orthogonal contrasts provide strong evidence ($p < 0.001$) for main effect of Zn fertilizer on grain Zn concentration as well as variety effect, some evidence for an interaction of Fe and Zn ($p < 0.05$), moderate evidence ($p < 0.01$) for a difference between the 30% NPKS and NPKS at recommended rate treatments (see Supplementary materials). Therefore, the application of Zn fertilizer significantly improves the grain Zn concentration while it is slightly dependant on the level of Fe fertilizer. Location was a source of variation for grain Zn concentration with Arsi Negelle having larger grain Zn concentration than Gojjam (see Supplementary materials). Despite soil Zn concentrations were significantly different among slope positions, slope (having smaller variance [0.3155] than block, location and season) was not the major source of variation of grain Zn concentration (see Supplementary materials). Therefore, block within the farm, slope position and season shows a negligible effect on grain Zn concentration.

Grain Fe concentration was significantly different ($p < 0.001$) between fertilizer treatments. NPKS shows the lowest ($53.25\,\mathrm{mg\,kg^{-1}}$) and Zn-only fertilizer having the highest ($60.2\,\mathrm{mg\,kg^{-1}}$) grain Fe concentration (Figures 4.1, 4.2). There was an average 21.4 and 17.8% enhancement of grain Fe concentration due to application of $FeSO_47H_2O$ and $ZnSO_47H_2O$ (21.4%) and $FeSO_47H_2O$ alone (17.8%) fertilization, respectively (Figures 4.1, 4.2). There was no significant difference in grain Fe concentration between 30% NPKS and NPKS application at recommended rate. As for grain Zn concentration, irrespective of fertilizer treatment, location and slope position, grain Fe concentration significantly ($p < 0.01$) differed between the varieties ranging between 53.6 and $57.2\,\mathrm{mg\,kg^{-1}}$ (Figures 4.1, 4.2). However, there was no evidence for an effect of slope position and an interaction of fertilizer and variety effect (see Supplementary materials). Thus, the fertilizer main effect was replaced by four orthogonal contrasts (slope and interaction effect dropped).

The orthogonal contrasts provide strong evidence ($p < 0.001$) for interaction of Fe and Zn effect on grain Fe concentration, suggesting that the response to Fe strongly depends on the level of Zn fertilizer application (see Supplementary materials). The Analysis of Variance indicates moderate evidence ($p < 0.01$) for an effect of Fe fertilizer application as well as variety on grain Fe concentration (see Supplementary materials). Location was a source of variation in grain Fe concentration, with Gojjam having larger grain Zn concentration than Arsi Negelle (see Supplementary materials). However, block within the farm as well as farm within the location shows negligible effect on grain Fe concentration (see Supplementary materials).

4.4 Discussion

The present study investigated the impact of Zn and Fe agronomic biofortification on finger millet grain Zn and Fe concentrations. Irrespective of variety, location and slope positions, finger millet grain Zn and Fe concentration increased by 18.9–20% and 17.8–21.4%, respectively, as a result of Zn and Fe agronomic biofortification. This suggest that agronomic biofortification of finger millet can be an effective supplementary strategy to reduce Fe and Zn deficiency, if the increased Fe and Zn in grains are bioavailable. Our finding also indicates that finger millet's response to Zn and Fe agronomic biofortification significantly was affected by variety and location, but not slope positions.

Zinc fertilizer increased grain Zn concentration in finger millet

The current experiment shows strong evidence that Zn fertilization effectively enhances grain Zn concentration of finger millet. Joy et al. (23) reported an incremental effect of soil application of Zn fertilizer on Zn concentrations of maize (20%), rice (7%) and wheat (19%) in 10 African countries. In addition, an experiment by Botoman et al. (24) and Manzeke et al. (25) showed that maize grain Zn concentration was increased by 15 and 44.5% due to the application of 30 kg and 45 kg $ZnSO_4$ ha^{-1}, respectively. Similarly, 29% maize grain Zn enhancement was observed as a result of 50 kg $ZnSO_47H_2O$ ha^{-1} fertilizer application (26). Another research showed 17.7% increase in wheat Zn concentration in response to soil application of up to 37.5 kg $ZnSO_4$ ha^{-1} (27). Pal et al. (28) also reported 21.3% increase of Zn in chickpea through 25 kg $ZnSO_4$ ha^{-1}fertilizer application. Another study from India also showed an increase of 26.5% rice Zn concentration by applying 5 kg $ZnSO_4$ fertilizer per hectare (29). Similar increase in Zn concentration of wheat grain following soil Zn application was also seen in Australia (30), Turkey (31) and India (32). Contrary to the finds of previous studies reporting positive effects of nitrogen on grain Zn concentration (25, 26), in the present study nitrogen had no significant effect on grain Zn concentration.

Variations in relative response of grain Zn concentration to Zn agronomic biofortification was observed between and among the present and previous studies could be attributed to several factors including due to differences in crops' ability to relocalize and remobilize Zn into the grain (33, 34). Wu et al. (35) also reported that rice Zn concentration density in rice grain was closely associated with the ability to translocate Zn from old tissues to new tissues at both early and late growth stages and with phloem remobilization of Zn from leaves and stems to grains. Phloem mobility of each element greatly affects the amount of element remobilization in plants

(36), and Zn showed good remobilization *via* phloem mobility (35). In addition, grain Zn concentration also significantly decreased as a result of elevated CO_2 (37, 38) but increased as a result of heat stress (39), however the mechanism is still unknown. Another study reported that phosphorus can negatively affect Zn absorption by inhibiting colonization hence, impairs mycorrhizal uptake pathway (40, 41). Nitrogen significantly enhances Zn absorption in plants (42, 43), probably, by balancing charge which contribute to higher accumulation of cationic nutrients like Zn (42). Nitrogen also increases the activity of transporter proteins and nitrogenous compounds that helps to maintain Zn root uptake and shoot translocation (44, 45).

Iron fertilizer increased grain Fe concentration in finger millet

The current experiment shows strong evidence that Fe fertilization effectively enhances grain Fe concentration of finger millet. There is no available data on agronomic biofortification of Fe on finger millet results, however, on other crops available research showed a significant increase on wheat due to the application of Fe fertilizer. Results of a greenhouse experiment reported a 19.4% increase in grain Fe concentration due to soil application of 10 mg of $FeSO_4$ kg^{-1} of soil (42) and 43% increase in response to soil application of 75 $FeSO_4$ kg ha^{-1} (27).

Pahlavan-Rad and Pessarakli (46) observed a 36% grain Fe concentration increase due to 1% of $FeSO_4$ foliar application at stem elongation and flowering stages. Manzeke-Kangara et al. (47) also reported about 83% increase in finger millet grain Fe concentration due to foliar Fe-EDTA application at vegetative growth and flowering stage.

Agronomic biofortification of Fe is less well studied compared to Zn. Fe biofortification only moderately increase grain Fe concentration as compared to Zn biofortification. This might be associated to the fact that Fe has poor phloem mobility (48, 49). Also, when applied to calcareous soils, Fe is rapidly converted into unavailable forms (48, 49).

Interactions between Zn and Fe fertilizers on finger millet grain Zn and Fe concentration

Enhancement of 13 and 5.5% grain Fe and Zn concentration, respectively, were observed due to Fe and Zn interaction effect. Similarly, Pahlavan-Rad and Pessarakli (46) also reported an 8 and 13% increase of wheat grain Fe and Zn concentration, respectively due to Fe and Zn interaction. Zinc fertilizers also resulted in Fe accumulation in soybean roots and increased root to fruit Fe translocation in tomato plants (50). These might be due to both Zn and Fe deficiency in plant is signaled by the same gene (51, 52). Also, Fe-and Zn-regulated transporter encoding genes expression in roots and shoots is induced at the transcriptional level by Zn and/or Fe availability (53–55), which suggests these genes may control the uptake and homeostasis of Fe and Zn (56). However, additional experiments are required to understand the mechanism of Zn and Fe interactions.

Effect of finger millet genotype and response to Zn and Fe fertilizer on grain Zn and Fe concentration

Response to agronomic biofortification of Zn and Fe in current experiment was significantly influenced by finger millet variety. In the present study, Diga-01 variety accumulated the lowest Zn concentration while Meba variety had the highest Zn. Though, the genetic variability in mineral concentration of finger millet has been previously reported (57), there is no available data on the finger millet's genetic response to Zn and Fe fertilization. However, Wissuwa et al. (58) from Philippines showed that rice genotypes differ greatly in their response to foliar Zn treatments. Similarly, significant varietal response to the soil application of Zn fertilizer on rice grain Zn concentration was reported (59). Despite a similar root uptake rate or shoot accumulation of minerals, genotypic differences influence grain mineral concentrations (60).

Stability of a trait over different locations is an important factor in breeding programs (61). When a highly promising genotype for grain Zn and Fe concentration is identified, special attention should be paid to how the grain Zn and Fe concentration of that genotype vary with soil type (62). In the current experiment Meba and Urji finger millet varieties exhibited stability over the two locations on grain Fe and Zn concentration, respectively.

Effect of location on grain Zn and Fe concentration in finger millet

Location had significant effect on grain Zn and Fe concentration of finger millet. Similarly, their subnational scale data from Ethiopia and Malawi, Gashu et al. (5) reported location as a source of variability in cereal grain mineral concentration.

4.5 Conclusion

The application of 25 kg $ZnSO_47H_2O$ and 20 kg $FeSO_47H_2O$ per hectare along with recommended rate of NPKS enhances grain Zn and Fe concentration of finger millet and can offer an effective option to increasing Zn and Fe concentration among consumers. However, social acceptability of agronomically biofortified food and technological and economic feasibility of application of mineral blended fertilizers and social acceptability of agronomically biofortified foods in resource poor settings could be a challenge and warrants further studies are important. In addition, crop grains such as finger millet have high amount of fiber and antinutritional factors that reduce bioavailability of minerals hence, evaluation of the bio-accessibility and bioavailability of biofortified crops is essential. Furthermore, use of mineral fertilizers could be contamination risks to the environment from the minerals of interest and contaminants in the mineral mixtures. For example, Stuart et al. (10) reported extra addition of 28 tonnes of Cu to the soil each year from use of Cu fertilizer for cereal biofortification in parts of the United Kingdom.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DT, DG, EJ, RL, TA, and MB: conceptualization. DT: data collection, supervision, and Original draft preparation. DT and EB: sample preparation and analysis. DT, DG, and RL. Statistical analysis. DT, DG, EJ, MRL, EB, LW, TA, and MB: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

In principle the full model for these data would be:

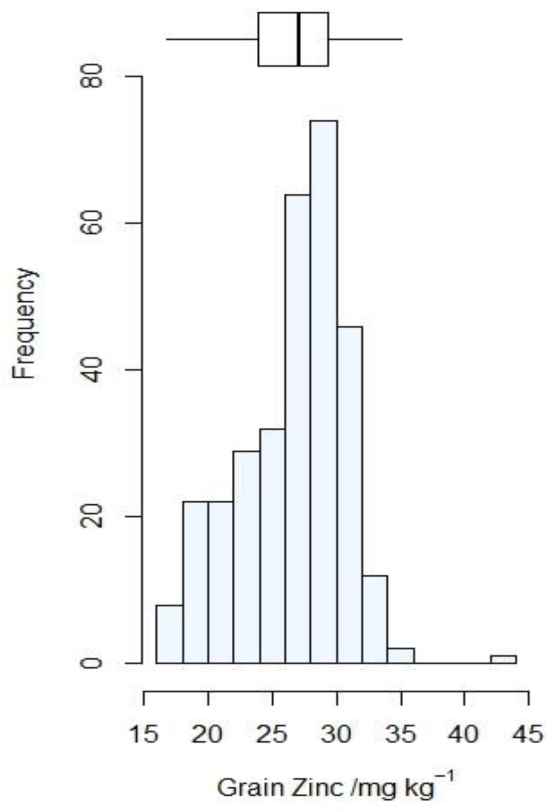
Fixed effects, slope, fertilizer, variety

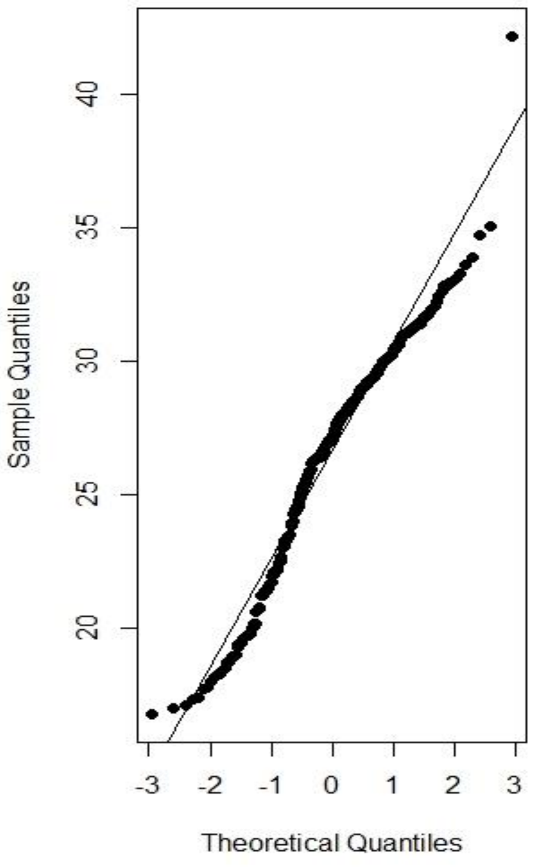
Random effects, Year and block within farm within location.

However, the way the randomization was done in the end, with all slope positions in a single farm within any location, it can be difficult to estimate the full model because of singularities. These will not automatically arise but will for some variables. Then, the strategy has been to run the full model where it can be, but to drop slope as a fixed effect where problems arise. Then one can examine the variance component for the farm random effect to get an idea of how important slope might be relative to other factors, as it will be a component of the between farm variance component in models where it is not a fixed effect.

Zinc

Exploratory analysis of the raw data. Summary statistics are output in R

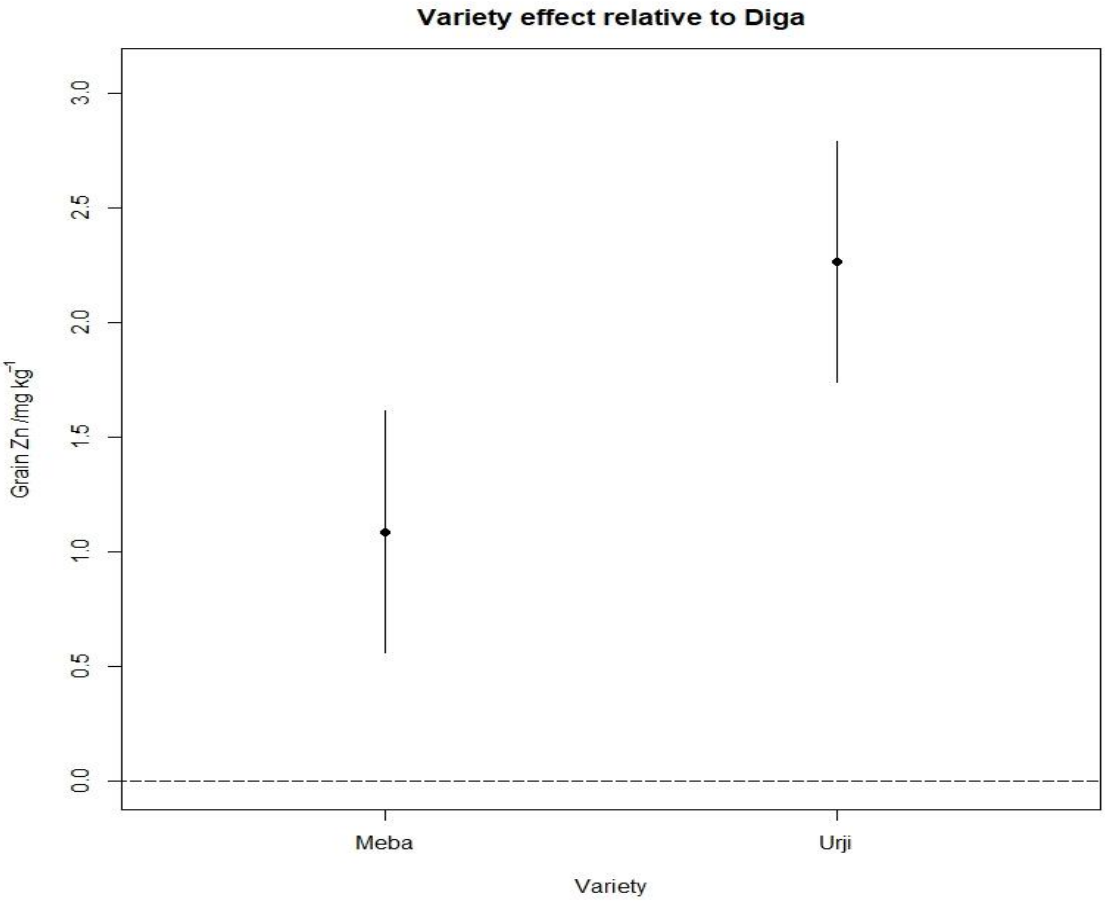




Analysis of variance for first basic model

```
> anova(model,refit=F)
Type III Analysis of Variance Table with Satterthwaite's method
                    Sum Sq Mean Sq NumDF  DenDF F value    Pr(>F)
FERTILIZER         167.939  41.985     4 274.68 11.3002 1.672e-08 ***
VARIETY            243.631 121.815     2 274.40 32.7865 1.707e-13 ***
FERTILIZER:VARIETY  27.619   3.452     8 275.85  0.9292    0.4926
```

Note there is evidence for differences among the fertilizer treatments, and between the varieties, but no evidence for an interaction. For this reason, further outputs are based on a model with this interaction dropped, specifically the following plots for fertilizer and variety effects



Finally, we run the model (interaction dropped) with the fertilizer main effect replaced by four orthogonal contrasts. These contrasts are as follows.

C1: The comparison between the mean grain Zn for the 0.3NPKS treatment and all the treatments with NPKS at recommended rate.

C2: Within the full NPKS rate, the Fe main effect (difference between treatments with Fe and no Fe)

C3: Within the full NPKS rate, the Zn main effect (difference between treatments with Zn and no Zn)

C4: The Fe/Zn interaction: does the response to Zn depend on the level of Fe?

```
> anova(model.r2)

Type III Analysis of Variance Table with Satterthwaite's
method

         Sum Sq Mean Sq NumDF  DenDF F value    Pr(>F)

C1       35.635  35.635     1 282.63  9.6062 0.0021348 **

C2       11.903  11.903     1 282.93  3.2088 0.0743113 .

C3       49.416  49.416     1 282.97 13.3211 0.0003125 ***

C4       18.363  18.363     1 282.98  4.9502 0.0268756 *

VARIETY 265.974 132.987     2 282.49 35.8493 1.334e-14 ***

---

Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

So, we can see strong evidence for a main effect of Zn fertilizer, some evidence for an interaction of Fe and Zn, moderate evidence for a difference between the 30% NPKS and NPKS treatments and a strong variety effect. This interprets the two key plots above.

The summary function applied to this final model allows to examine the variance components for each random effect.

Note that the between farm variance is small (0.3155), smaller than season, location or block effects. This suggests that slope position is not a major source of variation in grain Zn content.

```
Random effects:

 Groups                       Name        Variance Std.Dev.

 BLOCK_ID:(FARM_ID:LOCATION) (Intercept)  0.3290  0.5736

 FARM_ID:LOCATION            (Intercept)  0.3155  0.5617

 LOCATION                    (Intercept) 19.4043  4.4050

 YEAR                        (Intercept)  0.8907  0.9438

 Residual                                 3.7096  1.9260
```

Supplementary table 4.1. Effect of fertilizer type and finger millet variety on biofortified finger millet Zn concentration

Factors	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr (>F)
Fertilizer	167.939	41.985	4	274.68	11.3002	1.672e^{-08} ***
Variety	243.631	121.815	2	274.40	32.7865	1.707e^{-13} ***
Fertilizer: variety	27.619	3.452	8	275.85	0.9292	0.4926

Significance codes: *** < 0.001

Supplementary table 4.2. Type III Analysis of Variance with Satterthwaite's method for grain Zn concentration

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
C1	35.635	35.635	1	282.63	9.6062	0.0021348**
C2	11.903	11.903	1	282.93	3.2088	0.0743113
C3	49.416	49.416	1	282.97	13.3211	0.0003125***
C4	18.363	18.363	1	282.98	4.9502	0.0268756*
Variety	265.974	132.987	2	282.49	35.8493	1.334e^{-14}***

Significance codes: *** <0.001; **< 0.01; *< 0.05

C1: The comparison between the mean grain Zn for the T4 (30% NPKS) and all the treatments (NPKS at recommended rate)

C2: Within the NPKS at recommended rate, the $FeSO_47H_2O$ main effect (difference between treatments with $FeSO_47H_2O$ and no $FeSO_47H_2O$)

C3: Within the NPKS at recommended rate, the $ZnSO_47H_2O$ main effect (difference between treatments with $ZnSO_47H_2O$ and no $ZnSO_47H_2O$)

C4: The $FeSO_47H_2O$/$ZnSO_47H_2O$ interaction: does the response to $FeSO_47H_2O$ depend on the level of $ZnSO_47H_2O$

Supplementary table 4.3. Variance components of random effects for grain Zn concentration

Groups	Name	Variance
Block within the farm	Intercept	0.3290
Slope position	Intercept	0.3155

Location	Intercept	19.4043
Season	Intercept	0.8907
Residual		3.7096

Supplementary table 4.4. Effect of fertilizer type and finger millet variety on biofortified finger millet grain iron concentration, Ethiopia

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr (>F)
Slope position	140.01	140.01	1	2.985	1.9644	0.256013
Fertilizer	1781.52	445.38	4	274.420	6.2488	7.959e-05***
Variety	719.14	359.57	2	273.683	5.0448	0.007055 **
Fertilizer:variety	1021.69	127.71	8	274.800	1.7918	0.078574.

Significance codes: *** <0.001; **< 0.01; *< 0.05

Supplementary table 4.5. Type III Analysis of Variance with Satterthwaite's method for grain Fe concentration

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
C1	0.71	0.71	1	282.15	0.0097	0.9215000
C2	679.75	679.75	1	283.29	9.3327	0.0024654**
C3	17.22	17.22	1	283.29	0.2364	0.6272052
C4	987.87	987.87	1	283.13	13.5630	0.0002763***
Variety	738.34	369.17	2	281.78	5.0685	0.0068774**

Significance codes: ***< 0.001; **< 0.01; *<0.05

C1: The comparison between the mean grain Fe for the T4 (30% NPKS) and all the treatments (NPKS at recommended rate),

C2: Within the NPKS at recommended rate, the $FeSO_47H_2O$ main effect (difference between treatments with $FeSO_47H_2O$ and no $FeSO_47H_2O$),

C3: Within the NPKS at recommended rate, the $ZnSO_47H_2O$ main effect (difference between treatments with $ZnSO_47H_2O$ and no $ZnSO_47H_2O$), and

C4: The $FeSO_47H_2O$/$ZnSO_47H_2O$ interaction: does the response to $FeSO_47H_2O$ depend on the level of $ZnSO_47H_2O$

Supplementary table 4.6. Variance components of random effects for grain Fe concentration

Groups	Name	Variance
Block within the farm	Intercept	12.16
Farm within the location	Intercept	21.51
Location	Intercept	172.56
Residual		72.84

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Chapter 5: Agronomic biofortification of zinc fertilizer enhances zinc bioavailability of finger millet

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Abstract

Agronomic biofortification of zinc (Zn) might be a useful strategy to increase the bioavailabilty of minerals like Zn by reducing phytate besides increasing the grain mineral concentration as well as yield. Therefore, the aim of the present study is to investigate the impact of Zn agronomic biofortification of grain Zn and phytate concentration as well as Zn bioavailability expressed as total available Zn (TAZ). The experimental design had 9 treatment combinations comprising 3 finger millet varieties and 3 fertilizer treatments: (T1) 20 kg ha^{-1} $FeSO_4$ + 25 kg ha^{-1} $ZnSO_4$ + NPKS; (T2) 25 kg ha^{-1} $ZnSO_4$ + NPKS; (T3) NPKS were applied to the soil. Then, finger millet samples were analysed for Zn and phytate concentration and then total available TAZ was calculated. The result indicated that fertilizer treatment showed a significant ($p < 0.001$) variation in grain Zn and phytate concentration ranging from 26.3 to 36.3 mg kg^{-1} and 284 to 362.8 µg g^{-1}, respectively. Similarly fertilizer treatment showed a significant ($p < 0.001$) variation in TAZ ranging from 0.51 to 2.57 mg 300 g^{-1}. Meba genotype shows a significant variation in grain Zn ($p < 0.01$) and phytate ($p < 0.001$) concentration as well as TAZ ($p < 0.01$) ranging from 26.3 to 33.8 mg kg^{-1}, 284 to 360.6 µg g^{-1} and 0.51 to 1.875 mg 300 g^{-1}, respectively. The result suggested that finger millet that biofortified with Zn have the potential to contribute from 77.14 to 100% for adult men and from 108 to 140% for adult women per 300 g to the recommended daily intake of Zn. Therefore, Zn agronomic biofortifiaction on finger millet could help to combat Zn deficiencies among the society special where finger millet cultivated as a staple crop.

Key words: *Agronomic biofortification, mineral bioavailability, finger millet, phytate, zinc*

5.1 Introduction

Soil application of Zn fertilizer to crops reported to enhance grain Zn concentration as well as yield (1, 2, 3, 4, 5) and dietary Zn intake as well as plasma Zn concentration (6). Joy *et al.* (7) and Zhang *et al*. (8) also reported the potential of Zn-fertilisers to alleviate human dietary Zn deficiency and reduction of DALYs in African and China. However effectiveness of soil Zn application is greatly affected by minerals interaction such as Zn and phosphorus (P) which reduces Zn transfer from soil to the plant. Moreover, bioavailability of Zn from biofortified crops is greatly affected by grain accumulation of antinutrients specifically phytate.

Phytate in plant is the major storage form of phosphorus in plant tissues, particularly bran and seeds (9) which constituted the major portion up to 82% of total P (10). Phytate is known to form complexes with metals like Zn, iron (Fe) as well as calcium (Ca) and hinder their bioavailability in the gastrointestinal tract and considered as antinutritional factor in food (11). Therefore, reducing the plant P will indeed reduce phytate, whose primary purpose in plant is P storage, which in turn enhances the bioavailability minerals like Zn.

Interaction between P and Zn in plants have long been recognized and well documented. Studies reported that P negatively affect Zn absorption by inhibits colonization resulting in impaired mycorrhizal uptake pathway in plants (12, 13). On the other hand multiple studies reported that the P deficiency in soil results in higher accumulation of Zn whereas Zn deficiency in soil leads to higher accumulation of P in plant (14, 15, 16). These findings suggest that the agronomic biofortification with Zn might also be a useful strategy to reduce the antinutritional factors like phytate and thus increase the bioavailabilty of minerals like Zn besides increasing the grain mineral concentration as well as yield. To our knowledge, there is no available previous data on finger millet for Zn fertiliser effect the potential bioavailability Zn. Therefore, the aim of the present study is to invetigate the impact of soil application of Zn on finger millet Zn and phytate concentration as well as Zn bioavailability.

5.2 Materials and methods

5.2.1 Field experiment

Agronomic biofortification experiments with Zn fertilizers were carried out in farmer fields, in one *Woreda*/District in the Oromia region, Ethiopia: Arsi Negelle (7° 19’ 38’’N 38° 38’ 54’’E). According to the agro-ecological classification of Ethiopia, the study site is characterized as sub-humid midlands located between 1500–2300 m.a.s.l and receive an average annual rainfall of 800–1200 mm (17). The experiment consisted of 9 treatment combinations: 3 finger millet varieties (Dagi-01, black grain colour; Urji white grain colour; Meba, brown grain colour) and 3 soil-applied fertilizer treatments as indicated in Table 5.1. Since it is reported interaction of Fe and Zn has synergetic effect on grain Zn concentration (18, 19), soil application of combined Fe and Zn was used as a treatment.

A randomized completed block design (RCBD) was used with 4 replications. The plot size was 4 m x 4 m, with gangway between plots being 1 m while distance between block and the border were 0.5 m each. The finger millet was sowed on mid-June and harvested on the first week of November 2020 G.C.

Table 5.1. Elemental application of nutrients from fertilizer in kg per hectare

Treatments	Zn	Fe	N	P	S	K
T1	5.5	4	32.1	3.59	15.89	31.2
T2	5.5	-	32.1	3.59	7.64	31.2
T3	-	-	32.1	3.59	5.24	31.2

T1: 25 kg $ZnSO_47H_2O$, 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea
T2: 25 kg $ZnSO_47H_2O$, 131 kg NPS, 60 kg ha-1 K, 54 kg urea
T3: 131 kg NPS, 60 kg K, 54 kg urea

5.2.2 Sample collection

Soil samples for nutrient analysis were collected from a 60 m^2 circular plot in the experimental location. Five sub-sample sites were located, the first at the centre. Two sub-sample points were selected at locations on a line through the plot centre along the crop rows, and two on a line orthogonal to the first through the plot centre. The ‘long’ axis of the sample array (with sample locations at 5.64 and 4.89 m) was oriented in the direction of crop rows with the ‘short axis’ (with sample locations at 3.99 and 2.82 m) perpendicular to the crop rows. A single soil sub-sample was collected (20 cm deep) at each of the five sub-sample points with a Dutch auger with a flight of length 150 mm and diameter 50 mm. Any plant material adhering to the auger was carefully removed, and the sub-samples were aggregated and stored in a single bag. The detail procedure on soil sample collection, processing and mineral analysis is indicated by Gashu *et al.,* (20).

Matured and dried finger millet crop stalks were taken so that approximately 20–50% of the sample envelope was filled (dimensions 15 cm × 22 cm), with samples placed grain-first into the sample bag and the stalks were twisted off the grain heads and discarded. The crop samples were hand threshed to produce approximately 1 kg of grain and whole-grain samples were packed in sample envelope (21).

5.2.3 Sample preparation

Whole-grain samples were air-dried in their sample bags. Each sample was then ground in a domestic stainless-steel coffee grinder, which was wiped clean before use and after each sample with a non-abrasive cloth. All preparation was done away from sources of contamination by soil or by dust. A 20 g subsample of the ground finger millet was then shipped to the University of Nottingham. Soil samples were oven-dried at 40 °C for 24 –48 h depending on the moisture content of the soil. Preparation took place in a soil laboratory to avoid cross-contamination with grain samples. Plant material was removed from each soil sample, which was then disaggregated and sieved to pass 2 mm (21). This material was then coned and quartered to produce subsample splits. A 150 g subsample of soil was poured into a self-seal bag, labelled and shipped to the UK for analysis in the laboratories at the University of Nottingham

5.2.4 Soil and grain mineral analysis

The soil samples were digested using Aqua-Regia for mineral analysis (ISO 11466; ISO, 1995). A certified reference material (CRM Wageningen, WEPAL ISE-850, Calcareous Soil) was used to determine % recovery. Operational blanks were also analysed at the same time following the same procedure to determine limits of detection (LODs). The detailed procedure for soil mineral analysis is reported elsewhere (20, 22). Soil mineral concentration of experimental site is presented in Table 5.2. The recovery for all minerals is between the acceptable ranges (85 – 120%).

Hot plate acid digestion was employed for grain sample digestion using methods described in Kumssa *et al.*, (22). Briefly 0.2 g of finger millet grain samples were weighed in digestion tubes and placed into heating blocks (Multicube 48, Anton Paar Ltd, UK). Then, 8 mL of concentrated HNO_3 (trace metal grade, Fisher Chemical, USA) was added to each tubes and left for 30 minutes at room temperature. The samples were heated for 2 hours at 115°C, left to cool for 10 minutes, and the samples were diluted to 50 mL using milliQ water (18.2 MΩ cm; Fisher Scientific). A 1 ml aliquot was transferred into an inductively coupled plasma tube and diluted again to 10 ml using milliQ water. A certified reference material (CRM Wheat 1567b, National Institute of Standards and Technology, Gaithersburg, MD, USA) was used to determine % recovery. Operational blanks (n = 20) were also analysed to determine LODs. Soil and crop sample minerals concentration were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo Fisher Scientific, Bremen, Germany).

Table 5.2. Soil mineral concentration in mg kg^{-1} of experimental site

B	Mg	P	S	K	Fe	Zn
3.9±0.79	2052±47	2061±49	122.7±0.4	3227±185	26918±1149	89±7

5.2.5 Phytate analysis

Phytic acid was analysed using the Wade Reagent method, after Latta & Eskin (23) and modified by Vaintraub & Lapteva (24). For extraction of phytic acid 0.2 g flour samples were centrifuged at 3000 rpm for 1 hour after adding 10 mL of 0.2 M HCl. Then 3 mL of the supernatant and 2 mL of Wade solution were added and samples shaken to mix. Absorbance was measured at 520 nm using a UV-VIS spectrophotometer (Lambda 950, PerkinElmer, Waltham, USA) and the amount of phytic acid calculated and expressed in µg g^{-1}.

5.2.6 Potential bioavailability of grain Zn

The potential of Zn bioavailabilty in human was calculated using a mathematical model of Zn absorption in humans as a function of dietary Zn and phytate (25). The assumption in this model is that diet quantities of Zn and phytate are the primary factors determining Zn absorption in human (26). Additionally total daily absorbed Zn (TAZ) (mg Zn d^{-1}) is a function of total daily dietary phytate (TDP; mmol phytate d^{-1}) and total daily dietary Zn (TDZ; mmol Zn d^{-1}). Therefore, a quantitative value of Zn bioavailability was calculated using Miller *et al.*, (25) method as later modified by Hambidge *et al.*, (27), which is the trivariate model of Zn absorption in human.

Equation 1: Total daily absorbed Zn (TAZ)

$$= 0.5*[(A_{MAX}+TDZ+K_R)*(1+TDP/K_P)]-\sqrt{[(A_{MAX}+TDZ+K_R)*(1+TDP/K_P)]^2-4*A_{MAX}*TDZ}$$

Where: A_{MAX} (maximum Zn absorption) = 0.091, K_R (equilibrium dissociation constant of Zn-receptor binding reaction) = 0.680, and K_P (equilibrium dissociation constant of Zn-phytate binding reaction) = 0.033 related to Zn homeostasis in human intestine (27).

5.2.7 Statistical analysis

Data were analysed using SPSS software version 20. The laboratory analysis was done in triplicate and presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was calculated to compare Zn and phytate concentration as well as TAZ across finger millet genotypes as well as fertilizer treatments. Fertilizer treatment and genotype was treated as fixed effect where block within farm was treated as random effect. Skewness of the crop samples Zn and phytate concentration as well as potential Zn absorption data and histograms were examined.

5.3 Result

Finger millet genotypes response to Zn agronomic biofortification towards grain Zn and phytate concentration as well as TAZ are indicated in Tables 5.3. The recovery percentage for grain Zn concentration was 96.4 which is between the acceptable ranges (85 – 120). Irrespective of genotype, fertilizer treatment showed a significant ($p < 0.001$, supplementary material) variation in grain Zn and phytate concentration ranging from 26.3 to 36.3 mg kg^{-1} and 284 to 362.8 µg g^{-1}, respectively, where T1 and T3 possess the highest Zn and phytate concentrations, respectively (Table 5.3). Similarly fertilizer treatment showed a significant ($p < 0.001$) variation in TAZ ranging from 0.51 to 2.57 mg 300 g^{-1} where T1 and T3 showed the highest and the lowest result (Table 5.3). Though it is statistically insignificant, the result indicated the synergetic effect of Fe and Zn soil application on both grain Zn and phytate concentration (Table 5.3).

Finger millet genotype showed a significantly ($p < 0.001$) varied response to different fertilizer treatments in grain Zn and phytate concentrations as well as TAZ (Supplementary material). Diga-01 genotype shows a significant ($p < 0.001$) variation in grain phytate concentration ranging from 287 to 357 µg g^{-1} but variation was insignificant for grain Zn concentration and TAZ (Table 5.3). Similarly, Urji genotype shows a significant ($p < 0.001$) variation in grain phytate concentration ranging from 298 to 362.8 µg g^{-1} but variation was insignificant for grain Zn concentration and TAZ (Table 5.3). Meba genotype shows a significant variation in grain Zn ($p < 0.01$) and phytate ($p < 0.001$) concentration as well as TAZ ($p < 0.01$) ranging from 26.3 to 33.8 mg kg^{-1}, 284 to 360.6 µg g^{-1} and 0.51 to 1.875 mg 300 g^{-1}, respectively (Table 5.3).

On the other hand, single fertilizer treatment showed a significantly ($p < 0.001$) varied result for different genotypes (Supplementary material). T1 shows a significant ($p < 0.001$) variation in grain phytate concentration ranging from 287 to 314.6 µg g^{-1} but variation was insignificant for grain Zn concentration and TAZ (Table 5.3). T2 shows a significant ($p < 0.001$) variation in grain phytate concentration ranging from 284 to 313.3 µg g^{-1} but variation was insignificant for grain Zn concentration and TAZ (Table 5.3). However, T3 shows insignificant variation for grain Zn and phytate concentration as well as TAZ (Table 5.3).

Table 5.3. Effect of fertilizer treatment on grain Zn and phytate concentrations and total daily absorbed Zn (TAZ) of different finger millet genotype

Variety	Diga-01			Urji			Meba		
Fertilizer	T1	T2	T3	T1	T2	T3	T1	T2	T3
Grain Zn (mg kg^{-1})	31.87±0.78	33.36±3.3	28.92±1.07	34.06±2.22	32.59±1.59	29.09±1.22	32.9±0.9	31.77±0.55	27.93±1.62
Grain Phytate (µg g^{-1})	288.8±1.13	308.8±1.53	354.4±3.05	300.1±1.84	309.8±3.52	359.3±3.54	310.2±4.38	284.5±0.41	356.7±3.91
TAZ (mg 300 g^{-1})	1.642±0.16	1.96±0.68	1.037±0.11	2.1±0.47	1.792±0.33	1.065±0.25	1.856±0.19	1.622±0.22	0.84±0.33

5.4 Discussion

Finger millet plays a major role in food and nutrition security for millions of resource poor and tropical smallholding farming communities (28). However, the crop is reported to have low grain Zn concentrations ranging from 17.9 to 19.7 mg kg^{-1} (29) and high antinutrient content such as phytate ranging from 3363 to 14020 µg g^{-1} (30, 31). Though there is no available data on finger millet, agronomic biofortification of crops like wheat and maize with Zn fertilizer reported to have positive effect on grain Zn concentration as well as its bioavailability (32, 33). Therefore, the present study evaluated the impact of Zn agronomic biofortificaton on grain Zn and phytate concentration as well as TAZ of different finger millet genotypes.

The current study showed that agronomic biofortification of Zn on finger millet significantly increased grain Zn concentration and reduced phytate concentration, thus, subsequently improves Zn bioavailability expressed as TAZ which is consistent with a previous studies. For instance, Hussain *et al.*, (34) reported 24.3% reduction in wheat grain phytate concentration as well as 88% and 83.7% increases in grain Zn concentration and relative bioavailability of Zn expressed by TAZ, respectively, as a result of 18 kg ha^{-1} $ZnSO_47H_2O$ application. Liu *et al.*, (33) also reported a significant increase in grain Zn and relative bioavailability of Zn expressed by TAZ as a result of $ZnSO_47H_2O$ application up to 150 kg ha^{-1}. Erdal *et al.*, (35) also reported that soil application of 5.1 kg ha^{-1} elemental Zn on different wheat cultivars (n=20) increased grain Zn concentration up to 109% and decreased phytic acid concentration and estimated Zn bioavailability expressed as phytate:Zn molar ratios by 17.6% and 125%, respectively. Similarly, Bharti *et al.,* (36) reported 80% increase in grain Zn concentration and 23.2% and 73.4% decreases in phytic acid concentration and phytate:Zn molar ratio, respectively, of different wheat genotypes (n =10). Another study also indicated that grain phytate concentration and phytate:Zn molar ratio reduced by 17% and 52%, respectively, as a result of 2.5 kg ha^{-1} elemental Zn fertilizer application on wheat (37).

The beneficial effect of Zn agronomic bifortification on phyate and Zn bioavailability could be attributed to: a high-affinity P transporter proteins embedded in the plasma membrane of roots cell is responsible for P uptake (38). Though the expression of the genes that encode these transporters and their transcription is normally tightly controlled, under Zn deficiency this tight control is lost which results very high accumulation of P in barely and corn (39, 40, 41). Therefore, the presence of sufficient soil Zn helps to avoid excess uptake of P by the plant which in turn reduces the concentration of phytate whose primary role in plants is P storage. In barley Zn deficiency in the regulation of these genes was specific in that it cannot be replaced by others like manganese, nitrogen, and sulphur deficiencies (40).

On the other hand multiple studies reported that P fertilization effectively increased phytate concentration and reduced grain Zn concentration as well as Zn bioavailability. For example, application of 2 kg ha^{-1} elemental P fertiliser decreased grain Zn concentration up to 39% and increased P, phytate and phytate:Zn molar ratios by 17%, 19%, and 100%, respectively (42). Similarly, Imran *et al.* (32) reported that soil application of single P fertilizer decreases grain Zn concentration up to 5% while the combined application of P and Zn fertilizer increased grain Zn concentration up to 87% as compared to the control from their study on different maize genotypes. In addition, grain phytate concentration increased by 22% for single P fertilizer application, however, only 2.5% increase was observed for a combined P and Zn fertilizer applications. These studies suggested that the single application of P fertilizer effectively reduces grain Zn concentration as well as its bioavailability. The antagonistic effect of P on plant Zn uptake and concentration might be due to that P fertiliser decreased the colonisation of arbuscular mycorrhizal fungi (AMF) which assist the uptake of immobile nutrients such as Zn from soil to the plant (42). Therefore, a balanced or optimal application of P and Zn fertilizer might be beneficial in improving the grain Zn concentration as well as its bioavailability, since it is reported that even application of Zn fertilizer to the soils with sufficient Zn still reduced grain phytate concentrations and improves Zn bioavailability in wheat (43).

Contribution of finger millet biofortified with Zn to dietary Zn supplies were estimated assuming consumption three meals a day, each meal including a 100 g on dry basis. The recommended daily intake of Zn requirements is 2.1 mg day^{-1} for adult men and 1.5 mg day^{-1} for adult women (44). Therefore, assuming an adult person consumes a total of 300 g capita^{-1} day^{-1} on a dry basis and dietary Zn supply from finger millet ranges from 8.38 to 8.73 mg day^{-1} for control and ranges between 9.53 and 10.22 mg day^{-1} for fertilizer treatments. Therefore, a person consuming the above diet, will get from 0.84 to 1.07 mg Zn day^{-1} for control and from 1.62 to 2.1 mg Zn day^{-1} for biofortified finger millet. The result suggested that finger millet that biofortified with Zn have the potential to contribute from 77.14 to 100% for adult men and from 108 to 140% for adult women to the recommended daily intake of Zn. The significant increase in Zn bioavailability expressed by TAZ due to Zn agronomic biofortification was caused by not only the reduction of P/phytate concentration but also due to the increase in grain Zn concentration.

5.5 Conclusion

Agronomic biofortification of Zn on finger millet effectively reduced grain phytate concentration and enhances grain Zn concentration, thus, subsequently improves Zn bioavailability expressed as TAZ. However, the impact of Zn agronomic biofortification on Zn bioavailability significantly influenced by genotype. Therefore, soil application of combined 20 kg $FeSO_4 7H_2O$ and 25 kg $ZnSO_4 7H_2O$ per hectare could be a finest agronomic biofortification strategy to enhance Zn bioavailability of Urji and Meba genotypes in the study area and area with similar agro-ecologies. Moreover, soil application of 20 kg $ZnSO_4 7H_2O$ on per hectare on Diga-01 genotype could be a premium agronomic biofortification strategy to improve Zn bioavailability. Therefore, Zn agronomic biofortification on finger millet can offer an effective and immediate option to increasing the consumption of potentially bioavailable Zn among consumers where finger millet cultivated as a staple crop. Further study on *in vivo* Zn bioavailability from agronomically biofortified finger millet is strongly recommended.

Supplementary material

Supplementary table 5.1. Effect of fertilizer treatment on grain Zn and phytate concentration and TAZ

	Sum of Squares	Mean Square	NumDF	F value	Pr (>F)
Grain Zn (mg kg^{-1})	137.332	68.666	2	24.257	0.000*
Grain Phytate (µg g^{-1})	25476.964	12738.482	2	148.485	0.000*
TAZ (mg 300 g^{-1})	5.783	2.891	2	24.003	0.000*

Significance codes: *** < 0.001

Supplementary table 5.2. Fertilizer effect on grain Zn and phytate concentration and TAZ of Diga-01 genotype

	Sum of Squares	Mean Square	NumDF	F value	Pr (>F)
Grain Zn (mg kg^{-1})	21.397	10.698	2	2.539	0.134*
Grain Phytate (µg g^{-1})	9035.355	4517.678	2	1047.372	0.000*
TAZ (mg 300 g^{-1})	0.915	0.458	2	2.539	0.134

Significance codes: *** < 0.001

Supplementary table 5.3. Fertilizer effect on grain Zn and phytate concentration and TAZ of Urji genotype

	Sum of Squares	Mean Square	NumDF	F value	Pr (>F)
Grain Zn (mg kg^{-1})	25.03	12.515	2	4.206	0.051

Grain Phytate (µg g^{-1})	8061.97	4030.985	2	427.09	0.000*
TAZ (mg 300 g^{-1})	1.078	0.539	2	4.107	0.054

Significance codes: *** < 0.001

Supplementary table 5.4. Fertilizer effect on grain Zn and phytate concentration and TAZ of Meba genotype

	Sum of Squares	Mean Square	NumDF	F value	Pr (>F)
Grain Zn (mg kg^{-1})	31.863	15.931	2	12.762	0.002#
Grain Phytate (µg g^{-1})	10707.974	5353.987	2	463.943	0.000*
TAZ (mg 300 g^{-1})	1.346	0.673	2	12.940	0.002#

Significance codes: *** < 0.001, # <0.01

Supplementary table 5.5. Gonotypic response to Fe and Zn+NPKS fertilizer treatment on grain Zn and phytate concentration and TAZ

	Sum of Squares	Mean Square	NumDF	F value	Pr (>F)
Grain Zn (mg kg^{-1})	3.316	1.658	2	0.783	0.486
Grain Phytate (µg g^{-1})	915.879	457.939	2	57.549	0.000*
TAZ (mg 300 g^{-1})	0.148	0.074	2	0.784	0.486

Significance codes: *** < 0.001

Supplementary table 5.6. Gonotypic response to Zn+NPKS fertilizer treatment on grain Zn and phytate concentration and TAZ

	Sum of Squares	Mean Square	NumDF	F value	Pr (>F)
Grain Zn (mg kg^{-1-1})	3.562	1.781	2	0.39	0.688
Grain Phytate (µg g^{-1})	1638.663	819.332	2	165.35	0.000*
TAZ (mg 300 g^{-1})	0.158	0.079	2	0.399	0.682

Significance codes: *** < 0.001

Supplementary table 5.7. Gonotypic response to NPKS fertilizer treatment on grain Zn and phytate concentration and TAZ

	Sum of Squares	Mean Square	NumDF	F value	Pr (>F)
Grain Zn (mg kg^{-1})	1.575	.788	2	0.450	0.651
Grain Phytate (µg g^{-1})	48.894	24.447	2	1.195	0.195
TAZ (mg 300 g^{-1})	.064	0.032	2	0.447	0.653

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Chapter 6: Mineral bioavailability of agronomically biofortified with zinc and iron and fermented finger millet

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Abstract

Agronomic biofortification enhance crop micronutrient concentration while fermentation improves the bioavailability of micronutrients. Here the combined effect of Zn and Fe agronomic bifortification and traditional fermentation of finger millet on Fe, Zn and phytate concentration as well as bioaccessibility of Fe and Zn is reported. Five fertilizer treatments: (T1) 20 kg ha^{-1} $FeSO_4$ + 25 kg ha^{-1} $ZnSO_4$ + NPKS; (T2) 25 kg ha^{-1} $ZnSO_4$ + NPKS; (T3) 20 kg ha^{-1} $FeSO_4$ + NPKS; (T4) NPKS; (T5) 30% NPKS were applied to the soil. Then, finger millet samples were traditionally fermented from 0 to 94 hours at a room temperature. Samples were analysed for Fe, Zn and phytate concentration as well as bioaccessibility of Fe and Zn. The result indicated that finger millet grain Zn and Fe concentration increased by 21 and 20%, respectively, as a result of Zn and Fe agronomic biofortification. Bioaccessible fraction of Zn and Fe increased up to 81 and 88%, respectively, and phytate concentration reduced up to 49% as a result of fermentation. The result suggested that agronomically biofortified and fermented finger millet could potentially contributes up to 126% for men and 90% for adult women to the total absolute daily Fe requirements. Similarly, up to 179% for adult men and 251% for adult women of recommended daily intake of Zn could be fulfilled from agronomically biofortified and fermented finger millet. Therefore, it is possible to conclude that agronomic biofortifiaction on finger millet coupled with fermentation could help to combat Fe and Zn deficiencies among the society special where finger millet cultivated as a staple crop.

Key words: *Agronomic biofortification, bioaccessibility, iron, micronutrient deficiencies, zinc*

6.1 Introduction

Iron (Fe) and zinc (Zn) deficiencies are major public health concern and risk factors for global burden of disease (1, 2). In Ethiopia, a high prevalence of Zn (72%) (3) and Fe (34.4%) (4) deficiencies have been reported based on biomarkers. Iron and Zn deficiencies can lead to impaired physical growth and cognitive functions, reduced resistance to infections, metabolic disorders, and increased prenatal morbidity (5). Therefore, reducing the Fe and Zn deficiency should be given a due attention at national as well as global levels for the betterment of social and economic wellbeing since they are major impediment to socioeconomic development and contributes to a vicious circle of malnutrition, underdevelopment and poverty (6).

Deficiencies of Fe and Zn are more prevalent in developing countries because of the diet in those nations is dominated by monotonous cereal staple foods (7, 8). This is attributed to, beside cereal crops inherently have very low in cereal grain Zn and Fe (9), their highest mineral inhibitors content hiders the bioavailability of minerals (10). Thus, agronomic biofortifcation of Fe and Zn were suggested to improve grain mineral concentration (11) and different food processing techniques, especially fermentation, were suggested to increasing the bioavailability of minerals as potential strategies to improve human Fe and Zn status (12, 13, 14).

Finger millet’s (*Eleusine coracana* L.) ability to grown in arid regions, adapt to adverse climatic conditions, require minimal inputs and possess superior nutritional qualities makes it a major staple crop among tribal farming communities in developing countries (15, 16, 17). It is an indigenous crop to Ethiopia, and the sixth most important cereal crop after teff (*Eragrostis tef* Zucc.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), and barley (*Hordeum vulgare* L.). About 30 % of the global production is from Ethiopia where 1.2 M tons is produced every year at the national level (18). We have previously reported agronomic biofortification with Fe and Zn has significantly enhanced finger millet grain Fe and Zn concentration (11). Now we are reporting the potential bioavailability of the enhanced as well as inherent grain Fe and Zn as impacted by traditional fermentation.

6.2 Material and methods

6.2.1 Field experiment

The agronomic biofortification trials with Zn and Fe micronutrients were carried out at Gojjam (11° 41’ 54’’N 37° 29’ 79’’E) and Arsi Negelle (7° 19’ 38’’N 38° 38’ 54’’E foot slope) areas at farmers land. According to the classification of agro-ecological zonation of Ethiopia, both sites are characterized as sub-humid midlands located between 1500-2300 m.a.s.l. and receive an average annual rainfall 800-1200 mm (19). The experiment was laid out in randomized complete block design (RCBD) with factorial concept with 4 replications consisted of 5 levels of fertilizer application (Table 6.1).

Table 6.1. Elemental application of nutrients in kg per hectare

Treatments	Zn	Fe	N	P	S	K
T1	5.5	4	32.1	3.59	15.89	31.2
T2	5.5	-	32.1	3.59	7.64	31.2
T3	-	4	32.1	3.59	13.49	31.2
T4	-	-	32.1	3.59	5.24	31.2
T5	-	-	9.63	1.1	1.57	9.36

T1: 25 kg $ZnSO_47H_2O$, 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, and 54 kg urea ha^{-1}; T2: 25 kg $ZnSO_47H_2O$, 131 kg NPS, 60 kg ha^{-1} K, and 54 kg urea ha^{-1}; T3: 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, and 54 kg urea ha^{-1}; T4: 131 kg NPS, 60 kg K, and 54 kg urea ha^{-1}; T5: 30% of T4;

6.2.2 Sample collection and preparation

Finger millet samples were collected from the field and prepared in the laboratory following the method as described in Gashu *et al.*, (20). Briefly, about 1.25 kg of matured and dried fingers cut from the standing fingers millet using scissor. Then, the whole-grain samples were packed in sample envelope after the fingers were hand threshed in the laboratory to produce approximately 1 kg of grain. The whole-grain samples were air-dried in sample bags. The grain samples were ground in a domestic stainless-steel coffee grinder, which was wiped clean before use and after each sample with a non-abrasive cloth. All preparations were done away from sources of soil and dust contamination.

6.2.3 Fermentation process

Finger millet flour was mixed with ultra-purified water (pH: 7.2, conductivity at 25°C: 90 µS/cm) 1:2 ratio of weight base and let the dough to ferment for 4 days. Then similarly other four subsequent dough were produced using the successor dough as a backslop to start the fermentation. Then the final dough was used as a backslop for the experimental dough. The backslop fermentation was initiated without the addition of any starter culture. For experiments fermentation process finger millet flour from each treatment were mixed into dough with ultra-purified water with 1:2 ratio on weight basis. The dough was allowed to ferment naturally for 48, 72 and 96 hours (traditional fermentation took place from 2 to 4 days). The pH of the dough was measured at 48, 72 and 96 hours and the final pH of fermented dough ranged from 3.8 to

5.15. Then, the dough samples were dried using freeze drier (BI-FD-18N Lyophilizer Freeze Dryer, BR Biochem Life Sciences Private Ltd, India) and stored at -20ºC until further analysis.

6.2.4 Mineral analysis

Samples were acid digested in a hot plate as described in Gashu *et al*., (20). Briefly, about 0.2 g of sample was weighed into digestion tubes and placed into a heating block (Multicube 48, Anton Paar Ltd, UK). Concentrated HNO_3 (8 mL, trace metal grade, Fisher Chemical, USA) was added to each tube and left for 30 minutes at room temperature. The samples were then heated for 2 hours at 115°C and left to cool before dilution to 50 mL using MilliQ water (18.2 MΩ cm; Fisher Scientific). A further 1 in 10 dilution was undertaken immediately prior to analysis by inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo Fisher Scientific, Bremen, Germany). A certified reference material (CRM, Wheat 1567b, National Institute of Standards and Technology, Gaithersburg, MD, USA) was used to determine % recovery. Operational blanks (n=20) were analysed at the same time to determine the limit of detection (LOD) for each element.

6.2.5 Phytate analysis

Phytic acid was analysed using the Wade Reagent method, after Latta & Eskin (21) and modified by Vaintraub & Lapteva (22). For extraction of phytic acid 0.2 g flour samples were centrifuged at 3000 rpm for 1 hour after adding 10 mL of 0.2 M HCl. Then 3 mL of the supernatant and 2 mL of Wade solution were added and samples shaken to mix. Absorbance was measured at 520 nm using a UV-VIS spectrophotometer (Lambda 950, PerkinElmer, Waltham, USA) and the amount of phytic acid calculated and expressed in $\mu g\ g^{-1}$.

6.2.6 Mineral bioaccessibility test

The static *in vitro* digestion and dialysability of Fe and Zn were carried out according to the INFOGEST standardized consensus model (23) as latter modified by Muleya *et al.*, (24). Briefly, dried dough sample was mixed with Milli-Q water to make a 30% dry flour slurry. Then, for oral phase digestion, 2.5 g of sample flour slurry was mixed with 2.488 mL SSF which contains 0.012 mL $CaCl_2$ and 75 U mL^{-1} amylase. Then the mixture was incubated at 37 ºC, in a shaking water bath for 2 minutes after the pH is adjusted to 7.0. For gastric phase digestion, 5 mL of SGF which contain 2000 U mL^{-1} pepsin was added. Then, the mixture was incubated for 90 minutes after adjusting pH at 3.0. The dialysis bag which contain 17.5 mL of 0.05 M PIPES buffer (pH 6.7) was added to the sample digestion tubes incubated for a further 30 minutes. For the intestinal phase digestion, incubation continued for 2 hours after 5 mL of

SPF complete and 5 mL of SBF complete added pH is adjusted to 7.0. Then tubes were cooled using ice to stop enzyme activity. The dialysate was carefully transferred to clean storage tubes after removing the dialysis bag. Then, 4 mL of the dialysate was mixed with 2 mL of 50% HNO_3 and heated using microwave. Finally, bioaccessible fraction of Fe and Zn were analysed using ICP-MS.

6.2.7 Statistical analysis

Data were analysed using SPSS software version 20. The data were presented as mean ± standard deviation (SD). The analysis of variance (ANOVA) was performed to compare Fe, Zn and phytate concentrations, and bioaccessibility of Fe and Zn among control and biofortified and fermented finger millet samples. Mean comparison of data was done using Tukey's post-hoc analysis ($p < 0.001$, 0.01 and 0.05). Skewness of the samples Zn, Fe concentration and bioaccessibility of Fe and Zn data and histograms were examined.

6.3 Result

Finger millet response to Fe and Zn agronomic biofortification towards grain Fe and Zn concentration is indicated in Tables 6.2. The recovery percentage for grain Fe and Zn concentration was 91.0 and 96.4, respectively. Grain Zn concentrations ranging from 24.9 to 33 mg kg^{-1} while grain Fe concentration ranging from 48.1 to 66.8 mg kg^{-1}. For grain Fe concentration, T2 and T5 show the highest and lowest concentration, respectively, while for grain Zn concentration, T2 and T4 show the highest and lowest concentration, respectively. Phytic acid concentration of finger millet ranged from 334 to 396 µg g^{-1} (Table 6.2) depending on different fertilizer treatment where T5 and T2 show the highest and lowest concentration, respectively. Similarly, the bioaccessible fraction of Fe and Zn of finger millet ranged from 1.67 to 2.84 mg kg^{-1} and 1.68 to 9.15 mg kg^{-1}, respectively, (Table 6.2) depending on different fertilizer treatment where T3 for Fe and T2 for Zn show the highest while T4 for both show the lowest concentration.

Finger millet grain Fe and Zn showed a significant ($p < 0.001$) variation in response to Fe and Zn fertilization. There was a significant increase in grain mineral concentration, with 16.4, 21.2 and 13.2 % for Zn and 15.2, 17.4 and 20.2 % for Fe, in response to T1, T2 and T3, respectively. Similarly, finger millet grain phytate showed a significant ($p < 0.01$) variation in response to Fe and Zn fertilization. There was a significant reduction in grain phytate concentration, with 12.43, 14.41 and 10.83 % in response to T1, T2 and T3, respectively. On the other hand there was 21.35, 45.51 and 58.43 % increase for bioaccessible fraction of Fe in response to T1, T2 and T3, respectively, whereas there was 10.93, 65.85 and 36.07 % increase for bioaccessible fraction of Zn in response to T1, T2 and T3, respectively.

Phytic acid concentration of finger millet dough ranged from 213 to 353 µg g^{-1} (Table 6.2) depending on different fermentation period. There was a significant ($p < 0.001$) reduction in dough phytate concentration, with 35.5 and 29.6 % in response to 72 and 96 hours fermentation, respectively. The highest reduction of dough phytate concentration (49 %) was observed from 72 hours fermentation of T2.

Finger millet dough bioaccessible fraction of Zn and Fe ranged from 2.3 to 12.58 mg kg^{-1} and from 1.14 to 4.38 mg kg^{-1}, respectively, depending on different fermentation period (Table 6.2). There was a significant ($p < 0.001$) increase in dough bioaccessible fraction of Zn, with 74 and 65.4 % in response to 48 and 96 hours fermentations, respectively. Similarly, there was a significant ($p < 0.001$) increase in dough bioaccessible fraction of Fe, with 45.2 % in response to 72 hours fermentations. The highest increase in bioaccessible fraction of Zn (88.8 %) and Fe (81 %) were observed from 48 hours fermentation of T2 and 72 hours fermentation of T1, respectively.

Table 6.2. Phytate, total and bioaccessible fraction of Fe and Zn concentration of agronomically biofortified finger millet dough as affected by different fermentation period

Fermentation period	Fertilizer	Dough total Zn (mg kg^{-1})	Dough total Fe (mg kg^{-1})	Dough phytate (μg g^{-1})	Dough bioaccessible fraction of Zn (mg kg^{-1})	Dough bioaccessible fraction of Fe (mg kg^{-1})
0 hour	T1	30.5 ± 1.4^{a}	58.9 ± 1.7^{a}	346 ± 12^{a}	4.06 ± 0.23^{a}	2.16 ± 1.09^{a}
	T2	31.7 ± 1.5^{a}	60.45 ± 6^{a}	340 ± 6.1^{a}	6.07 ± 3.08^{a}	2.59 ± 0.03^{a}
	T3	29.6 ± 0.8^{a}	61.39 ± 4^{a}	351 ± 8.8^{a}	4.98 ± 1.92^{a}	2.82 ± 0.02^{a}
	T4	26.2 ± 1.3^{b}	51.1 ± 2.2^{b}	389 ± 6.1^{b}	3.66 ± 1.98^{a}	1.78 ± 0.11^{a}
	T5	26.7 ± 1.4^{b}	50.1 ± 1.3^{b}	390 ± 5.6^{b}	4.49 ± 2.21^{a}	2.16 ± 0.28^{a}
	Mean	28.9 ± 2.4^{A}	56.4 ± 5.4^{A}	363 ± 24^{A}	4.65 ± 0.93^{A}	2.3 ± 0.41^{A}
48 hours	T1	30.5 ± 1.4^{a}	58.9 ± 1.7^{a}	342 ± 1.8^{a}	7.59 ± 3.06^{a}	2.38 ± 0.79^{a}
	T2	31.7 ± 1.5^{a}	60.5 ± 6^{a}	319 ± 5.1^{a}	11.46 ± 1.12^{a}	4.19 ± 0.04^{b}
	T3	29.6 ± 0.8^{a}	61.4 ± 4^{a}	310 ± 17^{a}	8.12 ± 0.52^{a}	2.60 ± 0.28^{a}
	T4	26.2 ± 1.3^{b}	51.1 ± 2.2^{b}	343 ± 10^{a}	6.18 ± 1.35^{a}	2.81 ± 0.05^{ab}
	T5	26.7 ± 1.4^{b}	50.1 ± 1.3^{b}	322 ± 20^{a}	7.10 ± 1.71^{a}	1.99 ± 0.25^{a}
	Mean	28.9 ± 2.4^{A}	56.4 ± 5.4^{A}	327 ± 17^{A}	8.09 ± 2^{B}	2.79 ± 0.84^{AB}
72 hours	T1	30.5 ± 1.4^{a}	58.9 ± 1.7^{a}	246 ± 7.1^{a}	3.32 ± 1.02^{a}	3.91 ± 0.06^{a}
	T2	31.7 ± 1.5^{a}	60.5 ± 6^{a}	228 ± 15^{a}	5.60 ± 2.60^{a}	4.08 ± 0.30^{a}
	T3	29.6 ± 0.8^{a}	61.4 ± 4^{a}	240 ± 17^{a}	4.62 ± 1.69^{a}	3.82 ± 0.14^{a}
	T4	26.2 ± 1.3^{b}	51.1 ± 2.2^{b}	323 ± 5^{b}	3.91 ± 0.58^{a}	2.58 ± 0.47^{b}
	T5	26.7 ± 1.4^{b}	50.1 ± 1.3^{b}	303 ± 1.8^{b}	4.23 ± 2.05^{a}	2.31 ± 0.35^{b}
	Mean	28.9 ± 2.4^{A}	56.4 ± 5.4^{A}	268 ± 41^{B}	4.34 ± 0.85^{A}	3.34 ± 0.83^{B}
96 hours	T1	30.5 ± 1.4^{a}	58.9 ± 1.7^{a}	248 ± 3^{a}	8.19 ± 0.24^{a}	2.01 ± 0.25^{a}
	T2	31.7 ± 1.5^{a}	60.5 ± 6^{a}	248 ± 16^{a}	7.91 ± 0.63^{a}	2.01 ± 0.40^{a}
	T3	29.6 ± 0.8^{a}	61.4 ± 4^{a}	260 ± 4.3^{a}	7.53 ± 1.06^{a}	3.57 ± 0.64^{a}
	T4	26.2 ± 1.3^{b}	51.1 ± 2.2^{b}	330 ± 18^{b}	6.88 ± 0.35^{a}	1.88 ± 0.74^{a}
	T5	26.7 ± 1.4^{b}	50.1 ± 1.3^{b}	312 ± 7.1^{b}	7.95 ± 1.37^{a}	2.46 ± 0.28^{a}
	Mean	28.9 ± 2.4^{A}	56.4 ± 5.4^{A}	280 ± 37^{B}	7.69 ± 0.51^{B}	2.39 ± 0.7^{A}

T1: 25 kg $ZnSO_47H_2O$, 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T2: 25 kg $ZnSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T3: 20 kg $FeSO_47H_2O$, 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T4: 131 kg NPS, 60 kg K, 54 kg urea ha^{-1}; T5: 30% of T4. Results designated by different lowercases have significantly different response to different fertilizer treatment for single fermentation period. Results designated by different uppercases have significantly different response to different fermentation period.

6.4 Discussion

The present study investigated the combined impact of Zn and Fe agronomic biofortification on finger millet and fermentation on bioaccessibile fraction of Zn and Fe concentrations. Finger millet grain Zn and Fe concentration increased by 21 and 20 %, respectively, as a result of Zn and Fe agronomic biofortification. Bioaccessible fraction of Zn and Fe increased up to 81 and 88 %, respectively, as a result of fermentation. In addition phytate concentration reduced up to 49 % due to fermentation. This suggest that agronomic biofortification of finger millet combined with fermentation can be an effective supplementary strategy to reduce Fe and Zn deficiency.

The current experiment shows strong evidence that Zn and Fe fertilization effectively enhances grain Zn and Fe concentration and reduces phytate concentration of finger millet. Similar to the present study, many previous studies reported that an incremental effect of $ZnSO_4$ soil application (5 to 45 kg ha^{-1}) on Zn concentrations of cereals ranging from 15 to 45 % (25, 26, 27, 28, 29). Similarly, few previous studies reported that an incremental effect of $FeSO_4$ soil application (up to 75 kg ha^{-1}) on Fe concentrations of cereals ranging from 19 to 43 % (27, 30).

Reduction of phytate up to 49% were observed in the current study as a result of combined effect Fe and Zn agronomic biofortification and traditional fermentation. There is no available previous data on the combined effect Fe and Zn agronomic biofortification and traditional fermentation. However, similar studies on impact of fermentation like: Shumoy *et al.,* (31) from Ethiopia on teff, Gabaza *et al.,* (32) from Zimbabwe on finger millet and Greffeuille *et al.,* (33) from Benin on Maize, reported that 66%, 54% and 50% reduction of phytate concentration, respectively, as a result of traditional fermentation at a room temperature for 14 to 120 hours. The possible reason why such reduction of phytate as a result of fermentation were observed is that, during fermentation, endogenous phytase activity in cereals increases as a result of new synthesis and/or activation, resulting in reductions in inositol penta- and hexa-phosphates depending on the species and variety (34).

Enhancement of bioaccessible fraction of Fe up to 81% and Zn up to 88% were observed in the current study as a result of combined effect Fe and Zn agronomic biofortification and traditional fermentation. Similarly, Shumoy *et al.,* (31) from Ethiopia reported that the traditional fermentation of teff flour for 120 hours at 25ºC increased bioaccessible fraction of Fe up to two folds and Zn up to three folds. Another study from India also reported the increases in bioaccessibility of Zn by 50% and Fe by 127% in batters (made from rice and black gram with 3:1 ratio) as a result of 14 hours fermentation at a room temperature (34). A study from Benin reported Fe bioaccessibility increased nearly two fold as a result of 24 to 27 hours traditional fermentation of maize flour Benin (33). The current improvement in Zn and Fe bioaccessibilty could probably be attributed to that microbial fermentation, besides improving organoleptic properties, hydrolyse phytate by microbial phytase derived from naturally occurring microflora on the surface of cereal grains which in turn improve Fe and Zn solubility/bioavailability (35). Another possible reason that traditional fermentation improves Fe and Zn bioaccessibilty could also be attributed to the formation of organic acids during this process, which form soluble ligands with Fe and Zn (36), in fact it is observed that up to 2.1 unit reduction in the pH after fermentation in the current study.

The current result on bioaccessible fraction of Zn is in contrary to the report by Gabaza *et al.,* (32) where despite up to 54% destruction of phytate, 24 hours fermentation of finger millet flour at a room temperature showed no improvement in Zn bioaccessibility. This might be due to different reasons one in which might be the varietal difference (34). Shumoy *et al.,* (31) reported only two varieties of teff responded positively where the other two varieties were irresponsive in Fe bioaccessiblity to traditional fermentation, even though, all varieties showed a comparable reduction in phytate concentration. Another possible reason might be that even though there is a major destruction in phytate during fermentation, the residual inositol hexaphosphate (IP6) above 33 mg 100 g^{-1} might still play a major inhibitory effect on Fe bioavailability (37).

Contribution of fermented finger millet to dietary Fe and Zn supplies were estimated assuming consumption three meals a day, each meal including a 100 g on dry basis. The total absolute daily Fe requirements is 1.05 mg day^{-1} for adult men and 1.46 mg day^{-1} for adult women (38). Therefore, assuming an adult person consumes a total of 300 g $capita^{-1}$ day^{-1} on a dry basis and dietary Fe supply from finger millet is 15.3 mg day^{-1} for control and ranges between 17.7 and 18.4 mg day^{-1} for fertilizer treatments. Therefore, a person consuming the above diet, will get 0.534 mg Fe day^{-1} for control and up to 1.32 mg Fe day^{-1} for biofortified and fermented finger millet. The result suggested that agronomically biofortified and fermented finger millet from the current study could potentially contributes up to 126% for adult men and 90% for adult women to the total absolute daily Fe requirements. Similarly, the recommended daily intake of Zn requirements is 2.1 mg day^{-1} for adult men and 1.5 mg day^{-1} for adult women (38). Therefore, Zn supply from finger millet is 7.86 mg day^{-1} for control and ranges between 8.88 and 9.51 mg day^{-1} for fertilizer treatments. Therefore, a person consuming the above diet, will get 1.1 mg Zn day^{-1} for control and up to 3.77 mg Zn day^{-1} for biofortified and fermented finger millet. These result suggested that the agronomically biofortified and fermented finger millet from the current study could potentially contributes up to 179% for adult men and 251% for adult women to the recommended daily intake of Zn.

6.5 Conclusion

Agronomic biofortification of Fe and Zn on finger millet enhances grain Zn and Fe concentration. Furthermore, fermentation of agronomically biofortified finger millet effectively reduces phyate concentration and subsequently improves the bioaccessibity of Fe and Zn and can offer an effective option to increasing potentially bioavailable Zn and Fe consumption among the consumers. Therefore, agronomic biofortifiaction on finger millet coupled with fermentation could help to combat Fe and Zn deficiencies especially where finger millet cultivated as a staple crop. Further study on *in vivo* Fe and Zn bioavailability of biofortified and fermented finger millet is strongly recommended.

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Chapter 7: General Discussion, Conclusions and Recommendations

7.1 General Discussion

Finger millet plays a crucial role in food and nutrition security for millions of resource poor smallholding farming communities [1]. Breeding programs often focus on agronomic traits such as yield, drought tolerance and disease resistance [2]. However, it is also crucial to understand nutritional quality prior to genotype verification and official release of seeds. The present study investigate variation in the nutritional quality of 15 improved and released finger millet varieties. Genotype greatly influences proximate composition, mineral and antinutrient content of finger millet. Multiple previous studies reported significant variation in proximate composition, minerals, and antinutrients [2, 3, 4-9, 59-64].

Previous studies partly associate variation in mineral accumulation in grains to specific genes in the plant. For example, Mirza *et al.* [10] and Sharma *et al.* [6] reported that *EcCBP* and *EcCIPK7* genes and the activities of CaX exchanger and calmodulin (CAM) proteins in finger millet resulted high Ca accumulation. They also reported that these two genes were highly expressed in high calcium genotypes compared to medium and low calcium genotypes [10, 11]. Similarly, high accumulation of Fe and Zn in finger millet grains has been attributed to the regulation of potential key regulatory genes involved in Fe and Zn homeostasis particularly *EcFER1*, *EcIRT2*, *EcYSL2*, *EcZIP1* and *EcZTP29* genes [9]. Similarly, high concentrations of Se in finger millet has been also attributed to regulatory genes involved in Se homeostasis such as HOX4 and SPL genes [12].

Finger millet grain yield was enhanced by 20% due to soil application of $FeSO_4$ fertilizer irrespective of genotype, locations and slope position. However, different finger millet genotypes responds differently for both fertilizer treatment and location in respect to yield and yield traits. This suggests finger millet genotypes differ in their ability to remobilization and retranslocation of Zn and Fe deposited which plays a critical role in better partitioning of carbohydrates from leaf to reproductive parts affecting the yield and yield attributes. Therefore, the current finding should be taken into consideration in evaluating cereal genotypes for their response to agronomic biofortification.

Though there is no available previous report on the triple impact of Zn and Fe agronomic biofortification, genotype, and environment (location and slope position) on grain yield, some studies on a single factor reported a significant impact on cereals yield. For example, previous study shows that different finger millet genotypes responded differently to phosphorus fertilizer as well as locations in Kenya [13], to NPK fertilization in India [14], and to location in Ethiopia [15]. On the other hand, wheat and rice genotypes responded differently to Zn fertilization in Turkey [16] and to Zn fertilization as well as climate in India [17], respectively.

Application of Zn fertilizer, finger millet genotype, and an interaction of Fe and Zn had significant effect on grain Zn concentration. Similarly, application of Fe fertilizer and interactive effect of Fe fertilizer and finger millet genotype had significant effects on grain Fe concentration. Location but not slope position was also a source of variation for both grain Zn and Fe concentrations. Though there is no available data on impact Zn and Fe soil application on finger millet grain Zn and Fe concentration, current result is in line with previous reports on maize, wheat, rice and chickpea [18-20]. Interaction of Fe and Zn fertilization was reported to have synergetic effect on both grain Fe and Zn concentration in wheat [21]. Moreover, significant varietal response to the soil application of Zn fertilizer on rice grain Zn concentration [22] and location as a source of variability in cereal grain mineral concentration [23] were reported.

Different crops and genotypes have different ability to relocalize and remobilize mineral into the grain [24, 25]. For instance, Zn concentration in rice grain was closely associated with the ability to translocate Zn from old tissues to new tissues at both early and late growth stages and with phloem remobilization of Zn from leaves and stems to grains [26]. Phloem mobility of Fe and Zn greatly affects the amount of Zn and Fe remobilization as well as accumulation in plants [27]. Therefore, the current study suggested that finger millet, generally, have good ability to translocate Fe and Zn from old tissues to new tissues as well as a good remobilization ability of Fe and Zn *via* phloem mobility.

Besides improving grain mineral concentration, agronomic biofortification of crops like wheat and maize with Zn fertilizer reported to have positive effect on Zn bioavailability [28, 29]. Similar result was observed in the current where reduction in phytate concentration and subsequent increase in Zn bioavailability expressed as TAZ. Hussain *et al*. [30] and Liu *et al*. [31] also reported up to 83.7% increases in relative bioavailability of Zn expressed by TAZ as a result of soil application of Zn.

Soil application of Zn might affect P absorption through that a high-affinity P transporter proteins embedded in the plasma membrane of roots cell is responsible for P uptake [32] and plant control over these genes is lost under Zn deficiency and results very high accumulation of P [33-34]. On the other hand P fertiliser decreased the colonisation of arbuscular mycorrhizal fungi (AMF) which assist the uptake of immobile nutrients such as Zn from soil to the plant [35]. Therefore, a balanced and optimal application of P and Zn fertilizer might be beneficial in improving the grain Zn concentration as well as its bioavailability, since it is reported that even application of Zn fertilizer to the soils with sufficient Zn still reduced grain phytate concentrations and improves Zn bioavailability in wheat [36].

Phytate concentration was reduced up to 49% as a result of combined effect of fermentation and agronomic biofortification. Similar studies on impact of fermentation reported reduction in cereal phytate concentration from 50 to 66% as a result of traditional fermentation at a room temperature for 14 to 120 hours [37-39]. Endogenous phytase activity in cereals increases during fermentation as a result of new synthesis and/or activation, resulting in reductions in inositol penta- and hexa-phosphates depending on the species and variety [40].

Bioaccessibility of Fe and Zn significantly improved as a result of combined effect fermentation and agronomic biofortification. Similar studies reported the increament of bioaccessibility ranging from 50 to 200% for Zn and 127 to 300% for Fe as a result of traditional fermentation at a room temperature for 14 to 120 hours [38-40]. Microbial fermentation, besides improving organoleptic properties, hydrolyse phytate by microbial phytase derived from naturally occurring microflora on the surface of cereal grains which in turn improve Fe and Zn solubility/bioavailability [41]. Fermentation also improves Fe and Zn bioaccessibilty could also be attributed to the formation of organic acids during this process, which form soluble ligands with Fe and Zn [42] and up to 2.1 unit reduction in the pH after fermentation was observed in the current study.

7.2 Conclusions and recommendations

Finger millet proximate composition, mineral and antinutrient concentrations significantly varied by genotype but in general are good source of Ca and protein and a fair source of Fe and Zn. Moreover, all finger millet genotypes in present study possess excellent Zn, Fe and Ca bioavailability as evaluated using the molar ratio against dietary antinutrient (phytate and oxalate). The highest concentration and relative bioavailability of Ca in finger millet could play a role in combating Ca deficiency. It is recommended that the National breeding program of finger millet should consider nutritional qualities in addition to agronomic traits, since genotype significantly affect the nutritional quality of finger millet.

Genotype of finger millet greatly influences the response to agronomic biofortification of Zn and Fe fertilizer, which indicates the varied yield performance of the genotypes across environments (location and slope position). Therefore, evaluating genotypic response of finger millet to Zn and Fe fertilizer at different environments (location and slope positions) is crucial prior to scale up for mass production. Soil application of 20 kg $FeSO_47H_2O$ ha^{-1} for all genotypes and study areas as well as 20 kg $ZnSO_47H_2O$ ha^{-1} and combined 20 kg $FeSO_47H_2O$ ha^{-1} and 25 kg $ZnSO_47H_2O$ ha^{-1} on Urji and Meba genotypes, respectively, at Gojjam hill slope and area with similar agro-ecologies could be a premium agronomic biofortification strategy to improve finger millet grain yield. Future studies as well as development programs on agronomic biofortification should consider genotypic and environmental (location and slope position) effects beside the main fertilizer effect, which is a gap in current knowledge base.

Moreover, the soil application of 25 kg $ZnSO_47H_2O$ and 20 kg $FeSO_47H_2O$ ha^{-1} along with recommended rate of NPKS enhances grain Zn and Fe concentration of finger millet. Therefore, this strategy may offer an effective alternative to increasing consumption of Zn and Fe from cereals among consumers. However, social acceptability of agronomically biofortified food and technological as well as economic feasibility of application of mineral blended fertilizers in resource poor settings could be a challenge and warrants further studies.

Beside improving grain Zn concentration, agronomic biofortification of Zn effectively reduced grain phytate concentration, thus, subsequently improves Zn bioavailability expressed as TAZ. However, the impact is significantly affected by finger millet genotype and soil application of combined 20 kg $FeSO_47H_2O$ and 25 kg $ZnSO_47H_2O$ ha^{-1} for Urji and Meba genotypes as well as 20 kg $ZnSO_47H_2O$ ha^{-1} for Diga-01 genotype could be a finest agronomic biofortification strategy to enhance Zn bioavailability of in the study area and area with similar agro-ecologies. Therefore, Zn agronomic biofortification on finger millet can offer an effective and immediate option to increasing the consumption of potentially bioavailable Zn among consumers where finger millet cultivated as a staple crop.

Traditional fermentation of agronomically biofortified finger millet effectively reduces phyate concentration and subsequently improves the bioaccessibity of Fe and Zn. Fermentation of biofortified finger millet can offer an effective option to increasing potentially bioavailable Zn and Fe consumption among the consumers. Therefore, agronomic biofortifiaction on finger millet coupled with fermentation could help to combat Fe and Zn deficiencies especially where finger millet is cultivated as a staple crop. Further research on the *in vivo* Fe and Zn bioavailabity test is strongly recommended as every *in vitro* bioavailabity test must be confirmed with *in vivo* analysis.

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