Research Article

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Genetic Variability and Genotype X Environment Interactions Effect on Grain Iron (Fe) and Zinc (Zn) Concentration in Lentils and Their Characterization under Terai Environments of Nepal

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Abstract
Billions of peoples are directly affected from the micronutrient malnutrition called hidden hunger affecting one in three people. Micronutrient Iron (Fe), and zinc (Zn) deficiencies affect large numbers of people worldwide. Iron (Fe) deficiency leads to maternal mortality, mental damage and lower disease resistant of children. Likely Zinc (Zn) deficiency is responsible for stunting, lower respiratory tract infections, and malaria and diarrhea disease in human beings. Nepalese lentils are in fact rich sources of proteins and micronutrients (Fe, Zn) for human health and straws as a valuable animal feed. It has ability to sequester N and C improves soil nutrient status, which in turn provides sustainable production systems. Twenty five lentil genotypes were evaluated to analyze genotype × environment interaction for iron and zinc concentration in the grains. Analysis of variance (ANOVA) indicated that the accessions under study were found varied significantly (P=<0.001) for both seed Fe and Zn concentrations at all the three locations. Pooled analysis of variance over locations displayed highly significant (at P=<0.001) differences between genotypes, locations and genotype × location interaction for Zn micronutrient but insignificant genotype x location interaction was found in Fe micronutrient. Among 25 genotypes, the ranges for seed Fe concentration were 71.81ppm (ILL-2712)-154.03 ppm (PL-4) (mean 103.34 ppm) at Khajura, 79.89 ppm (ILL-3490)-128.14 ppm (PL-4) (mean 95.43 ppm) at Parwanipur, and 83.92 ppm (ILL-7979) -137.63 ppm (ILL-6819) (mean 103.11ppm) at Rampur, while the range across all the three locations was 82.53 ppm (ILL-7979) -133.49 ppm (PL-4) (mean 101.04 ppm). Likely the range for seed Zn concentration was 53.76 ppm (ILL-7723) – 70.15 ppm (ILL-4605) (mean 61.84 ppm) at Khajura, while the ranges for Parwanipur and Rampur were 54.21 ppm (ILL-7723) -91,94 ppm (ILL-4605) (mean 76.55 ppm) and 46.41 ppm (LG-12) – 59.95 ppm (ILL-4605) (mean 54.27 ppm) , respectively. The range across the three environments was 54.03 ppm (ILL-7723) – 75.34 ppm (HUL-57) (mean 64.22 ppm). Although both the micronutrients were influenced by environment, seed Fe was more sensitive to environmental fluctuations in comparison to seed Zn concentration. The G × E study revealed that it was proved that genotypes Sagun, RL-6 and LG-12 were more stable for seed Fe concentration and genotypes WBL-77, ILL-7164, RL-11 were found more stable for seed Zn concentration. In the AMMI analysis employing Gollob's test, first two PC explained 100% of the G × E variation. PC 1 and PC 2 explained 87.19% and 12.81% of total G × E interactions for Fe concentration and likely for Zn concentration; PC1 and PC2 explained 70.11% and 29.88%, respectively. The critical perusal of biplot revealed that Parawnipur locations was found to discriminating power for Fe concentration while for Zn concentration Khajura location was found to be most discriminative. The critical analysis of pedigree vis-à-vis micronutrient concentration did not reveal any correlation. This is probably the first report on iron and zinc concentration in lentil from Nepal.

Keywords: Lens Culinaris, Fe and Zn Concentration, G x E Interactions, AMMI Analysis

Introduction
Lentil (*Lens Culinaris* Medikus Subspecies Culinaris) is an annual diploid (2n = 2x = 14), self-pollinated and cool season legume crop grown in South Asia, Mediterranean region and North America. It is an important food legume with various uses as food and feed because of its protein-rich grains and straw. Globally, it is cultivated as a rainfed crop on 6.65 million hectares (m ha) area with 7.76 million tons production [1]. World lentil production has risen steadily by nearly eight times (843%) from an average of 0.92 mt in 1961-63 to 7.76 mt in 2017-18. This growth is primarily from an expanding harvested area from 1.64 m ha in 1961-63 to 6.65 m ha in 2017-

18. Additionally, it also reflects an improvement in productivity from an average yield of 560 kg ha-1 in 1961-63 to 1072 kg ha-1 by 2017-18. Lentils are consumed as a whole food in more than 54 countries, and annual world lentil production is approximately 7590761MT [1]. Approximately 54.4% of world's lentils are grown in South Asia, and nearly 1.5 billion people in this area consume 70% of the global lentil supply. Lentil in the diets of the people of South and West Asia, North and East Africa, and many developing countries provides nutritional security by supplying protein, Fe, Zn, and other macro- and micronutrients. The demands for lentil are expected to rise further due to population growth and rising income. By 2030, the world lentil consumption is estimated at 5.5 mt, being an increase of almost 2 mt from the present level [2]. In Nepal, lentil was grown in about 206969 ha with production of 254308 ton during 2017-18. This crop is mainly grown as rain fed crop in Dang, Banke, Bardia, Kailali, Bara, Parsa, Rauthat, Siraha, Saptari and Nawalparasi districts. It is the fifth most important crop grown after rice, maize, wheat & millet in terms of area [3]. Nepalese lentils are rich sources of proteins and micronutrients (Fe, Zn, Se), almost organic, fast cooking quality, small seeded, red cotyledons for human health and straws as a valuable animal feed [4]. Its ability to sequester N and C improves soil nutrient status, which in turn provides sustainable production systems. Globally, two billions peoples are directly affected from micronutrient malnutrition called hidden hunger affecting 1 in three people. Selenium (Se), iron (Fe), and zinc (Zn) deficiencies affect large numbers of people worldwide. Of the world's six billion people, 60% are Fe deficient, 30% are Zn deficient, and 15% are Se deficient [5]. Fe deficiency that lead to maternal mortality, mental damage of children and lower disease resistance [6,7]. The main role of Fe is to transport of O2 by RBC within hemoglobin molecules [7]. Fe def. anemia causes the most common nutritional disorder in the world. It is estimated to be 55.1% in children under five, 31% in school age children, 63.5% in pregnant women, and 35% in manual laborer (Directorate of Public Nutrition, 1993). Iron deficiency is more prevalent and vulnerable in developing countries like Nepal where around 23% peoples are suffering from the Fe deficient. Likely Zn deficiency is responsible for stunting, lower respiratory tract infections, and malaria and diarrhea disease in human beings. Approximately 1.4% (0.8 million) worldwide deaths (diarrhea, pneumonia, and malaria in children) were attributed to Zn def [8]. The main role of Zn is in several metabolic pathways including protein synthesis, cellular growth and differentiation [9,10]. It is reported that Zn deficiency cause anorexia, depression, altered reproductive biology, gastro-intestinal problems and impaired immunity [11]. Therefore, Fe and Zn are two essential nutrients for human nutrition. Iron is an integral part of protein involved in oxygen transport (hemoglobin, myoglobin), energy metabolism (cytochromes) and steroid and xenobiotic metabolism (cytochromes) [12]. Zinc is found in some 300 enzymes and is necessary for DNA replication, protein synthesis, reduction of free radical formation, proper sense of taste in food, and protection against brain tumors [13,14]. Indeed, micronutrient deficiency is caused due to non/poor availability of minerals in the diet. Hidden hunger is caused by a lack of vital minerals and vitamins in the diet. Harvest Plus focuses on three critical micronutrients recognized by the World Health Organization as most lacking in the diets of the poor (Vitamin A, Zinc & Iron). Dietary diversification, supplements and fortified foods are the important means to reduce micronutrient malnutrition.

Bio fortification is a process of increasing the levels of vitamins and minerals in food crops through plant breeding or agronomic practices, without compromising yield. When eaten regularly, bio fortified foods measurably improve nutritional status and health. Bio fortification is cost-effectiveness, highly sustainable and feasible [15]. Lentil is useful for human, animal and soil health. Lentil is important source of energy, protein, carbohydrate, fibre, mineral, and vitamin, antioxidant compound. It is a good source of vitamin A, potassium, vitamin B complex and iron. Within the existing food systems, micronutrient dense (Fe, Zn, and b-carotene) lentil cultivars will have a major effect in mitigating micronutrient deficiency syndromes, and the rural poor are the target group. A number of lentil cultivars have superior nutrient profiles, creating opportunities to enhance bio fortification and bioavailability by extensive induction of nutritional and anti-nutritional traits [16]. Also lentil has an excellent macro and micronutrient profile and favorable levels of mineral bioavailability enhancing factors. Lentil is cheap source of quality protein for poor and vegetarians [17,18]. The United Nations declaration of the 2016 International Year of Pulses recognized the role of pulses in human and animal health, environmental sustainability and economic benefits [19,20]. It has been documented that a huge population, particularly women and children in South Asia and sub-Saharan Africa, suffer from Fe, Zn, and Vitamin A deficiencies. To mitigate the problem of micronutrient malnutrition in developing countries under the Harvest Plus Program, in addition to improving yield, the aim of the study is to develop lentil cultivars with high contents of Fe and Zn [21]. The International Center for Agricultural Research in the Dry Areas (ICARDA) has a world mandate from CGIAR for lentil improvement. It delivers improved genetic material to the national programs of developing countries to develop new improved cultivars. Under the Harvest Plus Program, ICARDA conducts research to develop micronutrient-dense lentil genotypes to deliver to national programs. Its gene bank holds >11,000 accessions of diverse lentils collected from 72 countries, which are the key to genetic enhancement for micronutrients [22]. Out of 234 lines evaluated in Nepal, Fe content varied from 41 to 71 mg kg^{-1} in red lentils and from 59 to 89 mg kg-1 in green lentils, and Zn content in red lentils was 22 to 60 and 32 to 55 mg kg^{-1} in green lentils. These considerable variations in Fe and Zn contents suggest a scope for improvement in these traits [23]. Since in Nepal, no well report is available on estimation of Fe (iron) and Zn (Zinc) concentration and its stability across locations in lentil. The objectives of the study was to understand the genetic variability, effect of G x E interaction in lentil and to characterize and identify lentil germplasm accessions and breeding lines with stable concentrations of Fe and Zn in the seed which were also adapted environments in Nepal. The most widely used is the regression method, which is based on regressing the mean value of each genotype on the environmental index or marginal means of environments [24,25]. AMMI analysis provides a graphical representation (biplot) to summarize information on main effects and interactions of both genotypes and environments simultaneously [26].

Materials and Methods
Genetic material
The experimental material for the present study comprised of 25 lentil accessions originated from SAARC countries (14 accessions: Nepal-7, India-6, Bangladesh-1) and ICARDA (11 accessions). These accessions were tested in Genotypes x Environment bio fortification trial during 2016-17. These accessions were analyzed for seed Fe and Zn concentration (Table 1).

The test entries were evaluated at three locations in Grain Legumes Research Program (GLRP), Khajura, Banke at 810 37'' East longitudes and 280 06'' North latitude and an altitude of 181 meters above mean sea level, NMRP/NCRP, Rampur, Chitwan at 27o 40′ N latitude, 84o19′ E longitude at an altitude of 228m above mean sea level and RARS, Parwanipur at the latitude 270 4'40.9''N and longitude 84056'9.85''E as well altitude 75m above sea level during winter season 2016-17. The soils at Parwanipur are categorized as silt loam while at Rampur are well drained sandy loam and at Khajura are sandy to silty loam type. Surface soils samples were collected from each site of three terai environments (i.e. Regional Agricultural Research Station (RARS), Parwanipur, National Maize Research Program (NMRP), Rampur and Grain Legumes Research Program (GLRP), Nepalgunj before planting the G x E bio-fortified lentil trials. At least, soil samples were taken from representative 10 points of each replication under plough level soil depth i.e. 0-20 cm through using the soil auger and thoroughly mixed representative soil samples and made a composite sample of 0.5 kg of each replication i.e. three samples of each site had sent to the laboratory of Soil Science Division (SSD), Khumaltar for soil micronutrient Iron (Fe) and Zinc (Zn) analysis. The random method based on the variability of the land was used to collect soil samples. The different soil parameters tested as well as methods adopted to analyze the soil samples.
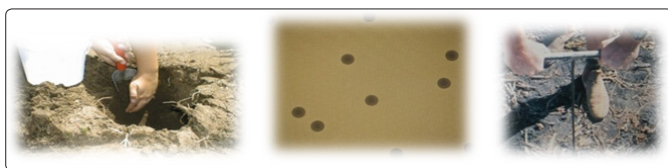

Figure: Distribution of Soil Samples Points during Soil Sampling

Soil Fe and Zn concentration at the experimental sites was estimated using standard procedures [27]. For the sample preparation methods; we require different sample preparation methods depending on the target suite of elements. Hence we used DTPA (Diethylenetriaminepentaacetic acid) extraction for the analysis of micronutrients (Fe and Zn) in soils and also need require extraction of macro nutrients Na, K, Ca and Mg for the determination of exchangeable cation with 1 M ammonium acetate. Descriptive statistics (mean, range, standard deviation, standard error, coefficient of variation) of soil parameters were computed using the Minitab 17 package. Rating (very low, low, medium, high and very high) of determined values were based on Soil Science Division, Khumaltar, Lalitpur, Nepal. The coefficient of variation was ranked according to the procedure of where, CV ≤ 25% = low variation, CV ≥25 ≤ 50% = moderate variation, CV >50% = high variation. For available soil Fe (ppm) and Zn (ppm) analysis, strictly followed protocols by DTPA [28,29].

The accessions were planted in Alpha-lattice design (5 x 5) with three replications per entry (6 rows per replication) with plant to plant spacing of 5cm and row to row spacing of 25cm. A unit plot comprise 2-meter length row with a plot size of (3 m^2). Five random plants from each plot were handled following the procedure suggested by Harvest Plus [21].

Determination for Lentil Grain Fe and Zn Contents
Atomic Absorption Spectrophotometer (AAS) was used for grain Fe and Zn concentration and model no. was Agilent 200. The grains were cleaned by mild hot water and dried at 35°C for five days in a contamination and corrosion free oven. From each sample, 5gm of grains were grounded manually into fine powder using mortar and pestle. Dust contamination was completely avoided. Biochemical analysis was carried out on triplicate ground samples of seeds from individual plant by digestion with 9:4 di-acid mixture (HNO$_3$: HClO$_4$) protocol using a microwave digestion system (Multiwave ECO, Anton Paar, les Ulis, France) followed by atomic absorption spectrometry (AAS) method using ECIL AAS (Perkin Elmer) as per the protocol described by Zarcinas et al., and Singh et al.. [27,30].

Methodology Details
Apparatus and Reagents
De-ionized water may be used. See 965.09A and B (See 2.6.01 and following
a. Potassium stock solution: 1000µgK/mL. Dissolve 1.9066 g dried (2h at 105°C) KCl in H$_2$0 and dilute to 1 L. Use following parameter for Table 965.09 (See 2.6.01): 7665 A (&66.5nm), air –C$_2$H$_2$ flame, and 0.04 -2 µg/mL range.
b. Calcium Stock solution – Prepare Ca stock solution and working standards as in 965.09B (see 2.6.01).
c. Cu, Fe, Mg, Mn, and Zn stock solutions – Prepare as in 965.09B (b), (c), and (e)-(g) (see 2.6.01).
d. Working standard solutions – Dilute aliquots of solutions, ©, with 10% HCl to make ≥4 standard solutions of each element within range of determination.

A. Preparation of Test Solutions
a. Dry Ashing: Accurately weigh 1 g test portion, dried and ground as in 922.02 (a) (see 3.1.02), into glazed, high form porcelain crucible, Ash 2 h at 500 °C and let cool. Wet ash with 10 drops H$_2$O and carefully add 3-4 mL HNO$_3$ (1+1). Evaporate excess HNO$_3$ on hot plate set at 100O-120°C. Return crucible to furnace and ash additional 1h at 500°C. Cool crucible, dissolve ash in 10 mL HCl (1+1), and transfer quantitatively to 50 mL volumetric flask.
b. Wet Ashing: Accurately weigh 1 g test portion, dried and ground as in 922.02 (a) (see 3.1.02), into 150mL Pyrex beaker. Add 10mL HNO$_3$ and let soak thoroughly. Add 3 mL 60% HClO$_4$ and heat on hot plate, slowly at first, until frothing ceases. Heat until HNO$_3$ is almost evaporated. If charring occurs, cool, add 10mL HNO$_3$, and continue heating. Heat to white fumes of HClO$_4$, cool, add 10 mL HCl (1+1), and transfer quantitatively to 50 mL volumetric flask.

B. Determination
a. To solution in 50 mL volumetric flask, add 10 mL 5% La solution, 965.09B (d) (see 2.6.01), and dilute to volume. Let silica settle, decant super mate, and proceed as in 965.09B (d) (see 2.6.01). Make necessary dilutions with 10% HCl to obtain solutions within range of instrument.

Standard	Potency (ppm)	Source	Lamp Serial no.
Iron(Fe)	1001	Merck	10N1544
Zinc(Zn)	998	Merck	11L292

Calculations
Element, ppm (µg/mL) x F/g test portion, Element % = ppm (µg/g) x 10^{-4} where F= (mL original dilution) x mL final dilution)/mL aliquot if original 50 mL is diluted.

Sample Dilution: SPL (1G) ---à 100 ml

$$\text{Iron (PPM)} = \frac{\text{Observed Conc.(ppm) x dilution x std potency}}{\text{Sample Weight (g)}}$$

Methodology Details for Grain Zn Concentration Analysis Using Atomic Absorption Spectrophotometer (AAS)

Principle

Test portion is wet or dry a shed. Residue is taken up in acid and diluted to optimum working range. A of this solution as determined by AAS at 213.8nm is converted to Zn concentration through calibration curve.

Reagents

(Use Pyrex glasses exclusively, clean thoroughly before use with HNO_3. If glass beads are used to prevent bumping, clean first with strong alkali followed by hot with HNO_3. Since pt used in laboratory may contain significant traces of metals, clean pt dishes by $KHSO_4$ fusion followed by 10% HCl leach.)

Zinc standard solution
Stock solution

500 µg/mL. Dissolve 0.500 g pure Zinc metal in 5-10mL HCl Evaporate almost to dryness and dilute to 1 L with H_2O. Solution is stable indefinitely.

Working solution

Dilute aliquots of stock solution with H_2SO_4 (1+49) or 0.1 M HCl (depending on method of ashing) to obtain ≥5 solutions within range of instruments. Prepare standards o-10 µg/mL range daily (Don't use <2 mL pipets or <25 mL volumetric flasks).

Acids

Reagent grade HNO_3, HCl, and H_2SO_4, test acids from freedom from Zn by AA measurement of appropriately diluted portion. If contaminated, purify HNO_3, and HCl by distillation. Further test purity of reagents and efficiency of cleaning by conducting blank determination by appropriate hashing methods.

Preparation of test Solution

Prepare representative test sample by mixing, blending or grinding.

Wet AsMShing

Accurately weigh into 300 mL or 500 mL Kjeldahl flask , test portion ≥10 g, estimated to contain 25-100 µg Zn (If material is liquid , evaporate to small volume) Add ca 5 mL HNO_3, and continuously heat until first vigorously reaction subsides. Add 2 mL H_2SO_4 and continue heating, maintaining oxidizing conditions by adding HNO_3 in small increments (large amounts may introduce Zinc) until solution is colorless. Continue heating until dense fumes of H2SO4 are evolved and all HNO_3 has been removed. Cool, dilute with Ca 20 mL H_2O filter through fast paper (prewashed) into 100 mL volumetric flask and dilute to volume with H_2O. Dilute further if necessary with H_2SO_4 (1+49) to attain working range of spectrophotometer.

Dry Ashing

Accurately weigh into clean pt dish representative test portion estimated to contain 25-100 µg Zn. Char under 1R lamp and ash at temperature ≤ 525 °C until C free.(Raise temperature of furnace slowly to 525°C to avoid ignition) Dissolve ash under watch glass

in minimum volume HCl (1+1). Add ca 20 mL H_2O and evaporate to near dryness on steam bath. Add 20 mL 0.1 M HCl and continue heating ca 5 minute. Filter through fast paper into 100 mL volumetric flask. Wash dish and filter with several 5-10 mL portions of 0.1 M HCl, cool, and dilute to volume with 0.1 M HCl. Dilute further, if necessary with 0.1 M HCl to attain working range of instrument.

Determination

Set instrument to previously established optimum conditions or according to manufacturer's instructions. Determine A of a shed solutions or dilution, and ≥5 standards within optimum working range, taking ≥ 2 readings (before and after test readings) Flush burner with H_2O and check 0 point between readings. Determine Zn content from standard curve obtained by plotting A against µg Zn/mL:

$$\text{Concentration Zn, mg/kg} = \frac{\text{µg Zn/mL from curve x (dilution factor, mL)}}{\text{g test portion}}$$

Statistical Analysis

The seed micronutrient data was subjected to analysis of variance (ANOVA) from the three environment trials for testing the significance of variation due to the genotypes, environment , years and their interaction for two seed quality traits using Genotype by Environment Analysis with R (GEA-R) and R-Stat version 3.5.3 statistical software. Stability analysis was performed following the Eberhart and Russell, 1966 regression model.

$$Y_{ij} = \mu + b_i I_j + \delta_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the mean for the genotypes i at location j; m is the general mean for genotype i; b_i is the regression coefficient for the ith genotype at a given location index, which measures the response of a given genotype to varying location; I_j is the environmental index, which is defined as the mean deviation for all genotypes at a given location from the overall mean; δ_{ij} is the deviation from regression for the ith genotype at the jth location and ε_{ij} is the mean for experimental error. A genotype having non-significant deviation from regression ($s2di = 0$) and unit regression coefficient ($b_i=1$) was considered as stable.

The analysis of variance of each location and combined analysis of variance over locations were done as per Gomez and Gomez [31]. The environmental mean was the mean of all genotypes in each environment. The pooled error was used to test the hypothesis that the mean square deviation did not differ significantly from 0 at 0.05 and 0.01% probability levels. The t-test employing the standard error of regression coefficient against the hypothesis that it did not differ from 1.0 was performed. It was assumed that genotype effects were fixed and year effects were random. Further, AMMI model, which combines ANOVA with principal component analysis (PCA) was used to study the agronomic nature of Genotype x Environment Interactions using (GEA-R) and R-Stat version 3.5.3. Plots were prepared as described by Zobel et al., using mean and first two PC scores [32]. The model AMMI uses the biplot constructed through the principal components generated by the interaction environment-genotype. If there is such interaction, the percentage of the two principal components would explain more than the 50% of the total variation; in such case, the biplot would be a good alternative to

study the interaction environment-genotype, Crossa [26].

The AMMI model is

$$Y_{ij} = \mu + \alpha_i + \beta_j + \sum_{k=1}^{t} \lambda_k \xi_{ik} \acute{\eta}_{jk} + \varepsilon_{ij}$$

where t is the number of SVD axes retained in the model, λ_k is the singular value for the SVD axis k, ξ_{ik} is the singular value of the genotype i for the SVD axis k, $\acute{\eta}_{jk}$ is the singular value of the environment j for the SVD axis k, and ε_{ij} is the error term of the models, where $\varepsilon_{ij} \sim iid\ N\ (0, \delta2)$.

Results and Discussion

In this study, 25 lentil accessions were evaluated at three diverse locations for grain Fe and Zn concentrations. Analysis of variance (ANOVA) indicated that the accessions under study were found varied significantly (P=<0.001) for both seed Fe and Zn concentrations at all the three locations. The ranges for seed Fe concentration were 71.81ppm (ILL-2712)-154.03 ppm (PL-4) (mean 103.34 ppm) at Khajura, 79.89 ppm (ILL-3490)-128.14 ppm (PL-4) (mean 95.43 ppm) at Parwanipur, and 83.92 ppm (ILL-7979) -137.63 ppm (ILL-6819) (mean 103.11ppm) at Rampur (Table 1), while the range across all the three locations was 82.53 ppm (ILL-7979) -133.49 ppm (PL-4) (mean 101.04 ppm). Likely the range for seed Zn concentration was 53.76 ppm (ILL-7723)-70.15 ppm (ILL-4605) (mean 61.84 ppm) at Khajura, while the ranges for Parwanipur and Rampur were 54.21 ppm (ILL-7723) -91,94 ppm (ILL-4605) (mean 76.55 ppm) and 46.41 ppm (LG-12)-59.95 ppm (ILL-4605) (mean 54.27 ppm) , respectively. The range across the three environments was 54.03 ppm (ILL-7723)-75.34 ppm (HUL-57) (mean 64.22 ppm). The environmental index for Fe and Zn in seed varied from location to location. It was –7.37 (Fe) and 2.61 (Zn) at Khajura, 0.776 (Fe) and -4.75 (Zn) at Parwanipur while at Rampur the index was 6.596 for Fe and 2.13 for Zn. The pooled ANOVA (Table 1) was carried out after ascertaining that the homogeneity of error variance using the Bartlett's test. G x E interactions were found to be significant (at P=<0.001) for Zn micronutrient but insignificant interaction was found in Fe micronutrient.

Table 1: Pooled Mean ANOVA for Seed Fe and Zn Concentration across the Environment over the Years Using R-Stat General Linear Model and GEA-R

Entry	Accessions	Fe (ppm)	Zn(ppm)
1	ILL-8006	90.63	64.98
2	RL-6	97.13	64.8
3	ILL-7715	94.23	65.68
4	ILL-7164	97.6	63.99
5	Khajura-2	114.66	62.78
6	Shital	96.21	67.08
7	Sagun	95.43	67.51
8	HUL-57	112	75.34
9	LG-12	87.23	64.18
10	RL-11	96.96	62.08
11	RL-4	84.77	63.39
12	Black Masuro	86.41	58.38
13	RL-79	121.59	65.12
14	ILL-6467	122.67	64.84
15	ILL-7979	82.53	63.47
16	RL-12	98.14	62.79
17	ILL-2712	87.68	65.56
18	PL-4	133.49	58.76
19	Simal	95.85	68.07
20	ILL-3490	91.74	57.06
21	ILL-6819	116.94	67.93
22	ILL-7723	94.09	54.03
23	WBL-77	87.77	63.22
24	ILL-4605	125.02	74.01
25	RL-49	104.86	60.41
	Mean	101.04	64.22
	P-value		
	Genotypes	0.007634 **	2.357e-05 ***
	Environment	9.012e-08 ***	6.982e-05 ***
	G x E interaction	0.784	0.009326 **
	CV%	22.87	10.98
	LSD	26.31	8.06
	PC1 (87.2%)	<0.001	<0.001
	PC2 (12.8%)	0.87	<0.001

Note: Significance Code: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 2: Micro Nutrients (Fe, Zn) Analysis Reports of Soil Samples

SN	Laboratory No. (sample code)	Description of soil	Soil testing site	Chemical properties	
				Fe (ppm)	Zn(ppm)
1	603	Rep I	RARS, Parwanipur	85.88	0.51
2	604	Rep II	RARS, Parwanipur	70.3	0.65
3	605	Rep III	RARS, Parwanipur	68.85	0.7
Mean				75.01	0.62
4	606	Rep I	NCRP, Rampur	48.76	0.53
5	607	Rep II	NCRP, Rampur	37.64	0.45
Mean					
6	608	Rep I	GLRP, Khajura	47.8	0.58
7	609	Rep II	GLRP, Khajura	58.3	0.7
8	610	Rep III	GLRP, Khajura	62.4	0.65
Mean				56.17	0.64
Grand Mean				58.13	0.58

Source: SSD, Khumaltar

Soil Analysis

The soil physical properties (texture, structure and color), pH, organic matter, primary nutrients, secondary nutrients and micronutrients (B, Fe, Zn, Cu and Mn) etc., are the key indicators of soil fertility [33]. These parameters indeed can predict the plant growth and development. Understanding of the soil fertility status is vital to develop proper soil management strategies helping in designing the planning crop cultivation in proposed area. Seed micronutrient concentration depends upon factors like soil type, soil fertility status, soil moisture, G x E, genotypic variation, soil profile, crop management practice and interaction among nutrients [34]. Soil Fe and Zn concentration at three locations were recorded as 75.01 ppm Fe and 0.62 ppm Zn at RARS, Parwanipur, 43.2 ppm Fe and 0.49 ppm Zn at NMRP, Rampur and 56.17 ppm Fe and 0.64 ppm Zn at GLRP, Khajura.

The available iron content ranged from 3.64 to 85.88 ppm with a mean of 58.13 ppm. This data indicated that it was found very high status of available iron in all three locations (Figure 1, Table 2). Likewise the available zinc content ranged from 0.45 ppm to 0.7 ppm with a mean of 0.58 ppm. This data clearly indicated that it was found low status of zinc in all three locations based on the soil parameters interpretations (Table 2 and 3, Fig ii). Thus, abovementioned micronutrient data indicated that the soil status exhibited variation for Fe and Zn concentration at the studied locations.

Figure 1: Distribution of soil iron in three locations (Left) and **Figure 2:** Distribution of soil zinc in three locations (Right)

Annex 1: Soil parameters interpretation at SSD, Khumaltar, 2016

SN	Soil parameters	Unit	Very low	Low	Medium	High	Very High
1	Available Fe	ppm	<5	5-10	10-16	16-25	>25
2	Available Zn	ppm	<0.5	0.5-1.0	1-3	3-6	>6

Table 3: Stability Parameters of 25 Lentil Accessions Studied Across Three Locations

Entries	Genotypes	Seed Zn			Seed Fe		
		Mean	bi	S²di	Mean	bi	S²di
1	ILL-8006	64.9867	1.2329	8.3611	90.64	1.58	-57.41
2	RL-6	64.8033	1.2191	-3.5762	97.14	1.06	-45.52
3	ILL-7715	65.685	1.3271	1.4367	94.23	1.29	-65.55
4	ILL-7164	63.9917	1.0759	-4.9447	97.6	1.91	-71.91
5	Khajura-2	62.78	1.113	-0.4699	114.66	0.47	-34.26
6	Shital	67.08	0.9633	13.266	96.22	3.2	128.84
7	Sagun	67.5117	0.8727	-3.3458	95.43	0.95	-70.78
8	HUL-57	75.3433	0.7763	356.94	112	0.56	312.09
9	LG-12	64.1883	1.1922	0.1648	87.23	1.04	-45.06
10	RL-11	62.08	0.9135	-2.5701	96.96	2.29	51.82
11	RL-4	63.395	1.1488	7.261	84.78	0.83	-78.51
12	Black Masuro	58.3867	1.1205	-2.971	86.42	1.18	-92.95
13	RL-79	65.12	1.4387	48.8843	121.59	-1.16	540.24
14	ILL-6467	64.845	1.0613	1.1921	122.67	1.85	3458.64
15	ILL-7979	63.4767	1.232	19.5391	82.53	-2.41	12.56
16	RL-12	62.795	1.2505	-3.956	98.15	2.01	-60.77
17	ILL-2712	65.5633	0.7389	26.6607	87.68	0.59	537.97
18	PL-4	58.765	0.3709	7.0387	133.49	1.42	480.94
19	Simal	68.0717	1.1652	0.507	95.86	2.47	425.18
20	ILL-3490	57.0667	0.0706	-3.5533	91.75	0.39	428.16
21	ILL-6819	67.9317	1.7811	-3.7489	116.94	-1.34	1026.56
22	ILL-7723	54.035	0.0071	-5.2636	94.09	2.25	-88.97
23	WBL-77	63.2283	1.0073	-5.1681	87.78	1	356.64
24	ILL-4605	74.0133	1.4419	-5.074	125.03	-0.17	153.39
25	RL-49	60.4167	0.4796	11.7699	104.87	1.75	46.4

Stability Coefficient Analysis

Four lentil accessions viz., Sagun, WBL-77, LG-12, RL-6 were identified as stable genotypes for seed Fe concentration based on regression coefficient (bi) while accessions Black Masuro, ILL-7723, RL-4, ILL-7164, Sagun, ILL-7715, RL-12, ILL-8006, RL-6, LG-12, Khajura-2 were identified as stable genotypes for seed Fe concentration based on the value of mean square deviation (S2di) while RL-11, Shital, WBL-77, LL-6467, ILL-7164 were identified as stable genotypes for seed Zn concentration based on regression coefficient (bi) while accessions ILL-7723, WBL-77, ILL-4605, ILL-7164, RL-12, ILL-6819, RL-6, ILL-3490, Sagun, Black Masuro, RL-11, Khajura-2 were identified as stable genotypes for Zn Concentration based on the value of mean square deviation (S2di) (Table 3). From the study, it was proved that genotypes Sagun, RL-6 and LG-12 were more stable for seed Fe concentration and genotypes WBL-77, ILL-7164, RL-11 were found more stable for seed Zn concentration.

The present study indicated higher sensitivity of seed Fe to environmental fluctuations. The study further revealed no correlation between seed Fe and Zn concentrations at all the three locations in the analyzed genotypes. This indicated the possibility of genetically improving the two target traits independent of each other using this specific set, including the most promising genotypes.

AMMI Analysis

Pooled analysis of variance (ANOVA) was carried out after analyzing the homogeneity of error variance using the Bartlett's test and the total iron and zinc concentration using GEA-R is presented in Table 1. There were significant differences among the genotypes, environments and G × E interactions for seed Zn concentration but there were not significant differences of G x E interactions for seed Fe concentration. Significant G × E interactions explained 41.55% and 17.12% of total sum of squares for Fe and Zn concentration respectively, which shows that although both the micronutrients are influenced by environments, seed Fe is relatively more sensitive to environmental fluctuations than seed Zn. Genotypic contribution towards total sum of squares were 54.66% for Fe and 16.14% for Zn concentration. Significant genotypic and environment differences suggested that genes necessary for micronutrient enrichment traits are available within the lens genome that could help for significant increases in seed Fe and Zn concentrations by recombination and directional selection. However, the ranges and means of seed Fe and Zn concentration varied widely at different locations due to the differences attributable to genotypes, environments as well as G × E interactions. In the AMMI analysis employing Gollob's test, first two PC explained 100% of the G × E variation. PC 1 and PC 2 explained 87.19% and 12.81% of total G × E interactions for Fe concentration and likely for Zn concentration; PC1 and PC2
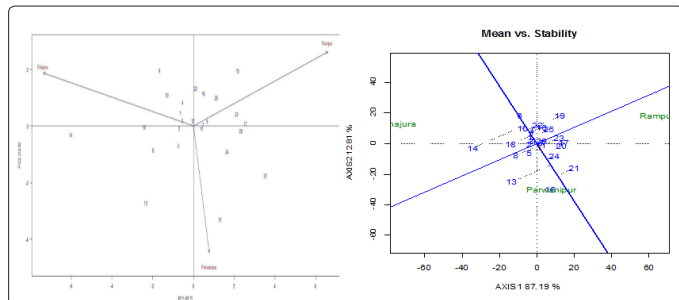
explained 70.11% and 29.88%, respectively.

Figure 3: AMMI (Additive Main Effects and Multicative Interactions Model) Plots for Seed Fe (a) Between Two PC and (b) Between Mean vs. Stability

The graphical method was employed by using two PC to investigate environmental variation and interpret the G × E interaction for Fe (Fig. 3a) and Zn (Fig. 4a) concentration [35]. Also, the AMMI biplot analysis between the mean vs. stability of G x E interactions (Figs. 3b & 4b) clearly indicated the distinct behavior of the environment. The lentil accessions closer to the origin of biplot were stable across the three test locations. For Fe concentration, the genotypes Black Masuro, Sagun, RL-4 and LG-12 were high and widely adapted for three locations. The genotype RL-79, ILL-6819 and ILL-6467 had high in Fe concentration but widely deviated from the origin of biplot that's why breeder cannot select it for hybridization program so ILL-7164 and RL-49 can be selected for hybridization program based on AMMI stability value and high Fe concentration basis whereas genotype simal, ILL6819, ILL-2712, ILL-3490 and WBL-77 showed highest Fe concentration but displayed more deviation from the origin of biplot (Fig. 3b).

Table 4: Stability Analysis Based on the AMMI Stability Value for Grain Fe Concentration

| Entry	ASV	YSI	rASV	rYSI	means
12	0.199	24	1	23	86.41
11	1.032	26	2	24	84.77
7	1.24	18	3	15	95.43
22	1.35	21	4	17	94.09
3	1.43	21	5	16	94.23
4	1.61	16	6	10	97.6
16	1.71	16	7	9	98.14
1	1.73	27	8	19	90.63
9	1.8	31	9	22	87.23
2	1.81	21	10	11	97.13
5	2.01	17	11	6	114.66
25	3.09	20	12	8	104.86
10	3.57	25	13	12	96.96
24	4.42	16	14	2	125.02
15	4.72	40	15	25	82.53
6	4.76	29	16	13	96.21
8	5.23	24	17	7	112
23	5.54	38	18	20	87.77
19	5.97	33	19	14	95.85
20	6.08	38	20	18	91.74
18	6.33	22	21	1	133.49
17	6.63	43	22	21	87.68
13	6.7	27	23	4	121.59
21	9.37	29	24	5	116.94
14	15.72	28	25	3	122.67
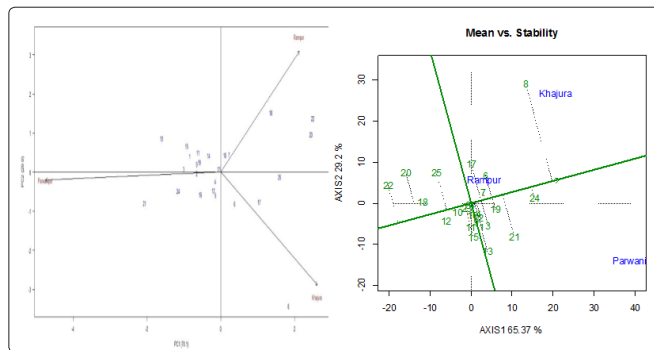
Figure 4: AMMI (Additive Main effects and Multiplicative Interactions model) Plots for Seed Zn (a) Between Two PC and (b) Between Mean vs. Stability

Table 5: Stability Analysis Based on the AMMI Stability Value for Grain Zn Concentration

Entry	ASV	YSI	rASV	rYSI	means
23	0.107	18	1	17	63.22
4	0.321	16	2	14	63.99
10	0.471	23	3	20	62.08
12	0.555	27	4	23	58.38
7	0.566	10	5	5	67.51
5	0.619	25	6	19	62.78
14	0.652	18	7	11	64.84
19	0.924	11	8	3	68.07
6	0.986	15	9	6	67.08
2	0.986	22	10	12	64.8
9	1.007	24	11	13	64.18
16	1.02	30	12	18	62.79
11	1.06	29	13	16	63.39
1	1.34	24	14	10	64.98
3	1.53	22	15	7	65.68
15	1.56	31	16	15	63.47
17	1.76	25	17	8	65.56
24	1.83	20	18	2	74.01
25	2.44	40	19	21	60.41
18	2.57	42	20	22	58.76
13	2.6	30	21	9	65.12
21	3.25	26	22	4	67.93
20	3.86	47	23	24	57.06
22	4.05	49	24	25	54.03
8	4.43	26	25	1	75.34

Similarly, the genotypes close to the X-axis (zero value of the first PCA of G x E interaction) were the most stable genotypes across environments for that particular trait. Genotypes WBL-77, RL-11 and Sagun are high in Zn concentration and are widely adapted for three locations (Fig 4a). From Fig. 4b, it is clear that the genotype ILL-7164, Khajura-2 and WBL-77 had less G x E interaction due to being closer to axis. The lines connecting the biplot origin and the margins for the environment are called environment vectors. The angle between two environment vectors is related to the correlation coefficient between them [36]. Based on the angles of the environment vectors the three locations have poor correlation between them for both the traits (Figs. 3a and 4a). Another observation related to length of the environment vector which depicts standard deviation within each environment. The critical perusal of biplot revealed that Parawnipur locations was found to discriminating power for Fe concentration (Fig 5a) while for Zn concentration Khajura location was found to be most discriminative (Fig 5b). The critical analysis of pedigree vis-à-vis micronutrient concentration did not reveal any correlation. The pedigree of genotypes showed high Fe (> 75 ppm) and Zn (> 60 ppm) concentration, here seed Fe concentration had specific parent contributing trait but seed Zn concentration did not indicate specific parent contributing to these traits. The present study clearly indicated the presence of significant variation for seed Fe and Zn concentration among the studied lentil genotypes and presence of genotype x environment interaction, besides identification of a set of promising and stable genotypes for seed micronutrient traits. The study further revealed no correlation between seed Fe and Zn concentrations at all the three locations in the analyzed genotypes. Among all genotypes ILL-7164 was most stable along with relatively better Zn concentration although HUL-57 had highest mean value with some G x E interaction. These genotypes are recommended for commercial cultivation and can also be effectively utilized in the recombination breeding programs to enhance genetic variability in lentil and also for generation of mapping population (s) for undertaking QTL analysis of seed micronutrient traits. This might be probably the first report of the iron and zinc study in lentil from Nepal.

Table 6: Biplot analysis for Grain Fe conc. across the environment over the years

Entry	type	Fe	PC1	PC2
1	GEN	90.63	-0.64	0.47
10	GEN	96.96	-1.3	1.089
11	GEN	84.77	0.393	-0.102
12	GEN	86.41	-0.029	0.184
13	GEN	121.59	-2.34	-2.71
14	GEN	122.67	-6.027	-0.311
15	GEN	82.53	1.29	-3.3
16	GEN	98.14	0.48	1.15
17	GEN	87.68	2.54	0.079
18	GEN	133.49	-2.42	-0.042
19	GEN	95.85	2.16	1.96
2	GEN	97.13	-0.693	-0.074
20	GEN	91.74	2.33	-0.17
21	GEN	116.94	3.52	-1.74
22	GEN	94.09	0.101	1.33
23	GEN	87.77	2.12	0.41
24	GEN	125.02	1.66	-0.89
25	GEN	104.86	1.122	1
3	GEN	94.23	-0.546	0.194
4	GEN	97.6	-0.529	0.84
5	GEN	114.66	-0.725	-0.694
6	GEN	96.21	-1.66	1.968
7	GEN	95.43	0.47	0.039
8	GEN	112	-1.97	-0.856
9	GEN	87.23	0.689	0.174
Khajura	ENV	103.34	-7.372	2.08
Parwanipur	ENV	95.43	0.776	-4.99
Rampur	ENV	103.11	6.596	2.91

Table 7: Biplot Analysis for Grain Zn Conc. across the Environment over the Years

| Entries	Type	Zn	PC1	PC2
1	GEN	64.98	-0.83	0.41
10	GEN	62.08	0.101	0.44
11	GEN	63.39	-0.61	0.497
12	GEN	58.38	-0.201	-0.46
13	GEN	65.12	-1.602	0.85
14	GEN	64.84	-0.32	0.41
15	GEN	63.47	-0.92	0.66
16	GEN	62.79	-0.55	-0.56
17	GEN	65.56	1.03	-0.76
18	GEN	58.76	1.35	1.52
19	GEN	68.07	-0.58	0.25
2	GEN	64.8	-0.64	-0.02
20	GEN	57.06	2.44	0.96
21	GEN	67.93	-2.06	-0.78
22	GEN	54.03	2.49	1.37
23	GEN	63.22	-0.04	0.07
24	GEN	74.01	-1.15	-0.47
25	GEN	60.41	1.59	-0.12
3	GEN	65.68	-1.002	0.07
4	GEN	63.99	-0.14	-0.22
5	GEN	62.78	-0.13	-0.58
6	GEN	67.08	0.38	-0.79
7	GEN	67.51	0.224	0.44
8	GEN	75.34	1.83	-3.42
9	GEN	64.18	-0.64	0.2
Khajura	ENV	61.84	2.61	-3.21
Parwanipur	ENV	76.55	-4.75	-0.22
Rampur	ENV	54.27	2.13	3.43
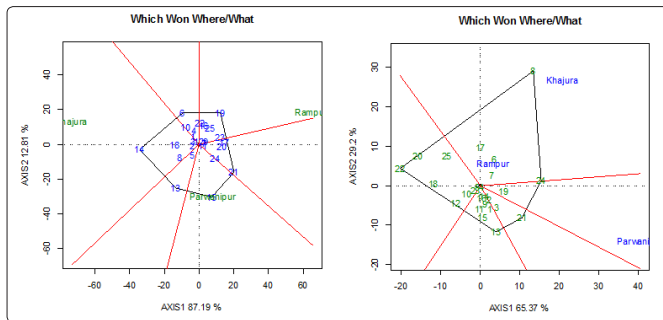
Figure 5: The which-won-where view of the GGE biplot to show which Fe concentration genotypes performed best in which environment (5a) and the which-won-where view of the GGE biplot to show which Zn concentration genotypes performed best in which environment (Fig 5b)

Discussion

The International Center for Agricultural Research in the Dry Areas (ICARDA) leads research to bio fortify lentils with higher levels of iron (Fe) and zinc (Zn). A large number of breeding lines, landraces, and released varieties have been analyzed for iron and zinc content. Iron content ranged from 42–132 ppm and zinc content ranged from 23–78 ppm. Ray et al., evaluated the availability of mineral content in the four commonly grown pulses, field pea (Pisum sativum L.), common bean (*Phaseolus vulgaris L.*), chickpea (*Cicer arietinum L.*), and lentil (*Lens culinaris* Medikus subsp. *culinaris*), in Saskatchewan, Canada, and found that they contain significant proportions of the recommended daily allowance for Mg, K, Fe, Zn, Mn, Cu, Se, and, in some cases, Ni. Lentil is among the earliest domesticated crops and plays an important role in human, animal, and soil health improvement [37]. Its seed is a rich source of protein and of minerals (K, P, Fe, and Zn) and vitamins, and its straw is a valuable animal feed. Iron and zinc were analyzed at Waite Institute, Australia; University of Saskatchewan, Canada; Indian Agricultural Research Institute, India; and North Dakota State University, USA. Several released varieties that possess high iron and zinc levels and have good agronomic performance have been identified. These varieties are in fast-tracking and include in Bangladesh: Barimasur-4 (86.2 ppm Fe), Barimasur-5 (86 ppm Fe, 59 ppm Zn), Barimasur-6 (86 ppm Fe, 63 ppm Zn), and Barimasur-7 (81 ppm Fe); in Nepal: Sisir (98 ppm Fe, 64 ppm Zn), Khajurah-2 (100.7 ppm Fe, 59 ppm Zn), Khajurah-1 (58 ppm Zn), Sital (59 ppm Zn), Shekhar (83.4 ppm Fe), and Simal (81.6 ppm Fe); in India: Pusa Vaibhav (102 ppm Fe); in Syria/Lebanon: Idlib-2 (73 ppm Fe) and Idlib-3 (72 ppm Fe); and in Ethiopia: Alemaya (82 ppm Fe, 66 ppm Zn) [21]. Identification of fast-track varieties, parents with high iron and zinc were developed and have been used in crossbreeding programs at ICARDA, Bangladesh Agricultural Research Institute (BARI), Nepal Agricultural Research Council (NARC), and Indian Agricultural Research Institute (IARI). Final, intermediate, and primary products have been developed and are under evaluation for yield traits and micronutrient levels. Identification of genetically fixed lines and germplasm with high levels of iron and zinc at ICARDA helped to develop new international nurseries for red lentils. Multi-location testing is strong, and varieties/advanced lines have been tested in Bangladesh, Ethiopia, India, Nepal, and Syria. Significant genotype-by-environment (GxE) interaction was observed in many cases; iron content is more sensitive to environmental fluctuations compared to seed zinc content. A few genotypes were identified with stable high-

iron and zinc contents (IPL 320, L4704). Still there are challenges to produce the sufficient quantities of quality seed of high-iron and zinc varieties and the development of high-yielding and high-micronutrient varieties with stable performance across environments. Abiding this, understanding of correlation of iron and zinc levels with other macro- and micronutrients in lentil seeds. High-iron and zinc content lines with excellent agronomic performance are selected from the ICARDA international nursery and national breeding programs of Bangladesh, India, and Nepal and are subject to regional testing. Iron lentils are also being tested in Ethiopia and Syria.

Lentils are an important cool-season legume crop for food and nutritional security of the Nepalese people. Till date, limited efforts have been made to assessment of Fe and Zn content [38]. Among the pulses, lentils are popular dishes in Bangladesh and Nepal; therefore, to assess their nutritive indicators, the present study was conducted to identify high-Fe and Zn-rich lentil accessions in Nepal. Based on a total of 25 accessions, analysis of variance (ANOVA) indicated that the accessions under study were found varied significantly (P=<0.001) for both seed Fe and Zn concentrations at all the three locations. There were significant differences among the genotypes, environments and G × E interactions for seed Zn concentration but there were not significant differences of G x E interactions for seed Fe concentration. The environmental index for Fe and Zn in seed also varied from location to location. De Araujo et al., also reported significant genotypic variation in common bean for micronutrients [39]. Based on the AMMI stability value and high concentration of Fe and Zn micronutrients, accessions ILL-7164 and RL-49 are recommended for commercial cultivation and can also be effectively utilized in the recombination breeding programs to enhance genetic variability and combined enhancement of nutrients in lentil. In addition, genotypes with high micronutrient levels might be suitable for studying the mechanisms of mineral element accumulation and transport. The mineral characteristics of crop plants depend on genetic and environmental factors. Variation in different germplasm for mineral characteristics also depends on the level of soil fertility, soil type, seed characteristics, seed composition, climatic factors, and others. The variance component estimates showed that genotype ´year interaction was not significant, thus indicating the genotypic stability over years for both the minerals. Significant genotype ´location interaction for Fe implied that there is location-specific adaptation of the genotypes. In a single-year multi location study, Kumar et al., also reported significant genotype, location interaction for both the micronutrients [40]. Although the accumulation of both the micronutrients was influenced by environment, the accumulation of seed Fe is more sensitive to environmental fluctuations in comparison to that of seed Zn. Similar findings were also reported in maize [41]. The Eberhart and Russell model revealed that the variation due to G x E (linear) was non-significant for seed Fe. However, the environment (linear) was significant for Zn (Table 3). In addition to this the factors like microclimatic effects and meteorological parameters play important role. Significant effects of genotype × location and genotype × year interaction for karnel Fe; and genotype × location × year interaction for kernal Zn were earlier reported in maize [41,42]. Despite various factors affecting seed micronutrient status including differential behavior of genotypes in locations, this study was successful in identifying promising lentil genotypes based on mean value of genotypes, regression values and deviations from regression. The importance of genotype x environment effects has also been assessed for nutrients and yield in other crops such as soybean, wheat, and

sweet potato, where the general conclusion was that the implications of genotype x environment interaction should be kept specific to the trait in designing the breeding program [43-46]. The available zinc content was found inadequate in the stations. Low organic matter status as well as negligence use about the need of boron and zinc for crops in the station might be the cause of low boron and zinc status. The available iron content was very high in the farm. The occurrence of high amount of primary and secondary iron minerals like olivine, siderite, goethite, magnetite etc. might be the cause of high content available iron [47]. High iron availability may show iron toxicity symptoms in crops [48]. The present study revealed no correlation between seed Fe and Zn concentrations at all the three locations in the analyzed genotypes, hence indicated the possibility of genetically improving the two target traits independent of each other in contradiction to earlier reports in different crops [49-52].

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