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Management of Iron and Manganese Toxicities of Lentil Crops Grown in Central Chile

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Abstract: Iron (Fe) and manganese (Mn) toxicity is a widespread problem in lentil production in the coastal dryland of Chile. Increasing the soil pH by liming with $CaCO_3$ or incrementing grain yields through nitrogen fertilization can help the plants to reduce metal concentration. Thus, the main objective of this work was to evaluate two different fertilization strategies (lime ($CaCO_3$) and nitrogen (N) additions) to reduce Fe and Mn toxicities in lentils. Lentils grown under field conditions with the highest Fe and Mn concentrations showed toxicity symptoms, but without grain yield reductions. In a pot experiment using the same soil as in the field with toxicity symptoms, the dry matter (DM) produced at the end of the trial was higher in the plants that received N while the lowest DM production was recorded in those plants treated with lime. In particular, higher root DM sustained the growth of the N-fertilized shoots, which also positively affected the grain yields being 33% higher than the control treatment (no fertilization addition). In the plants fertilized with N, the Fe and Mn levels in the shoots were lower than the control plants and those grown in soils treated with lime, but showed higher concentrations of Fe and Mn in roots. In parallel, roots exhibited high concentrations of Fe and Mn that were 13- and 9-fold higher than in the shoots. Additionally, a significant decrease of 29% in Mn concentration in the grains of plants treated with N was reported. Overall, our results suggest that an increase in DM of lentils by the addition of N can reduce the Mn concentration on leaves to a level that is likely under the threshold that causes toxicity in plant tissues. Finally, we conclude that the increase of Fe and Mn in the roots may be connected to the reduction of these metals on leaves.

Keywords: lentil production; manganese and iron toxicities; nitrogen fertilization; central Chile

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1. Introduction

Lentil production in Chile has decreased since the 1980s mainly due to international prices and the low technology used by local farmers which have negatively impacted both yields and the surface cropped with this legume species. However, the cultivation of lentils is part of local tradition and it is an essential component of the crop rotation system that is beneficial for local markets and farmers. Lentil production is mainly concentrated in marginal soils of the coastline of central Chile where the climatic conditions are benign due to the buffering effects of the Pacific Ocean. Specifically, Chilean lentil production areas are concentrated in Chanco, Curepto, and Navidad towns which are near the coastline (about 10 km). In the three locations, the soils are derived from sea deposits [1], and in all cases are nutrient depleted and degraded soils, either eroded or compacted [2]. Because of

these poor soil conditions, small farmers cultivate crops of low productivity such as lentils, mainly under rain-fed conditions and low fertilizers input [3,4]. Due to their nitrogen-fixing properties, crop rotations with legumes are essential to increase soil nitrogen (N) and soil organic matter (SOM). In this sense, local farmers cultivate lentils and chickpeas in rotation with cereals and pastures to feed farm animals as in other parts of the world [5,6], which is particularly beneficial for lentils grown in Mediterranean environments [6,7], such as central Chile.

Since the mid-1980s, farmers have reported the incidence of plants spotted with black and brown points [8]. After a thorough examination and chemical analysis of plant samples, it became evident that high concentrations of iron (Fe) and manganese (Mn) were responsible for the symptomatology [8–10]. Although Fe and Mn are essential nutrients for all plants, higher concentrations of these nutrients can result in toxicity symptoms [11]. Taking a sampling collection obtained in the main lentil production area in central Chile, the concentration of lentil leaves with black and brown spots reached up to 2120 mg/kg for Fe and 370 mg/kg for Mn, whereas healthy leaves maintained low levels of both elements (743 and 150 mg/kg, respectively) [9]. Normal concentrations of Fe and Mn are in the range of 200–1000 mg/kg and 100–175 mg/kg, respectively [10]. Normal foliar concentrations of Fe and Mn in other small grains species fluctuate between 25–250 mg/kg and 20–150 mg/kg, respectively [12]. The disorder is called locally "marea negra" (black tide) or "sereno" (sea mist, fog), and it is more prevalent in old leaves progressing to the young leaves, which produce initially black and brown spots, and necrosis and the consequent fall of leaves in cases of severe increase of Fe and Mn concentrations [8]. Because of this mineral toxicity, lentil crops reduce yield and the commercial quality of grains that have a negative impact on the farmers' economy.

Soils naturally contain significant amounts of Fe and Mn, Fe being one of the most abundant elements in the Earth's crust [13]. However, not all Fe and Mn in soils are available for plants. Soil conditions, particularly redox potential and pH, control Fe and Mn availability for the plant [14,15]. In an experiment with a soil where lentil plants were intoxicated with Fe and Mn, it was demonstrated that the main factor controlling the availability of these elements was the soil redox potential [16]. In this experiment, they showed that by changing the redox potential by replacing soil oxygen with nitrogen gas, there was an increase of Fe and Mn soil solubility of several folds. The Fe concentration was 0.47 g kg^{-1} under aerobic conditions, and it increased to 17.2 g kg^{-1} under anaerobic conditions, whereas there was an increase of 0.17 to 3.4 g kg^{-1} for Mn. Under normal soil pH and even with high pH values, the low redox conditions render Mn more available [17], similar to Fe [18]. The soils where lentil production in Central Chile is grown have pH ranging from slightly alkaline to acid (pH_w 7.0 to 5.5). Unlike calcareous and sodic soils that are very common in Mediterranean areas as in the Near East and North Africa (NENA) regions, where lentil production is common [19,20], the soils used by lentil farmers in Chile rarely show micronutrient deficiencies. Furthermore, soil organic matter (SOM) is in the range of 2% to 6% [21], which can also contribute to the chelation of Fe and Mn and their availability to the plant. Nonetheless, the SOM can also contribute as electron donors to increase ultimately the soil redox potential [22].

As a legume species, the roots of lentils can induce the reduction of Fe and Mn by the ferric reductase enzyme expressed on the epidermal cells of roots, which can greatly help the root system to absorb cation micronutrients [23–25]. Furthermore, because lentils can fix N_2 in symbiosis with rhizobia, there is an unbalanced uptake of cations that results in pumping H^+ to the rhizosphere to maintain the membrane potential in normal metabolic ranges, triggering the acidification of the rhizosphere [26,27].

Taking the soil processes together alongside with lentil´s roots metabolism, they can likely result in more Fe and Mn availability for the crop. Thus, the aim of this work was to assess two different fertilization strategies to reduce Fe and Mn toxicity in lentils. To reach this goal, we collected soil samples from a crop land with visible symptoms of Fe and Mn toxicity on lentil leaves and grains, and we carried out a pot experiment to evaluate

the effect of lime ($CaCO_3$) addition and N fertilization. Subsequently, we measured in the pot experiment plant productivity, root/shoot ratio (R/S), nodule number and Fe and Mn concentration of leaves, roots, and grains.

2. Materials and Methods

2.1. Field Sampling of Plant Material and Soils

Lentil grains and soil samples were collected from four sites in the Chanco area (Table 1), Maule Region, Chile, considering that brown and black spots on lentil leaves were observed on these samples. Furthermore, grain yield was determined by collecting samples of plants from eight mini-plots randomly distributed of 1 m^2 at the harvest time in each of the farmers' cropland (n = 8).

Table 1. Agronomic management conducted by four lentil farmers and their geographical locations in the town of Chanco, Maule region, central Chile.

Site and Geographic Location	* Sowing, Harvest and Threshing Date	Previous Crops	Sowing Surface (ha)	Nutrient Applied (kg ha^{-1})			
				N	P	K	S
Farmer 1 35°42′44.6″ S 72°31′07.0″ W	22 June 7 December 25 December	L/P/W	0.5	-	30	41.5	18
Farmer 2 35°42′44.6″ S 72°31′07.0″ W	22 June 7 December 25 December	W	0.9	-	30	41.5	18
Farmer 3 35°41′38.8″ S 72°30′10.6″ W	24 June 7 December 25 December	W	2.0	46	-	-	-
Farmer 4 35°40′50.1″ S 72°29′12.1″ W	24 June 7 December 25 December	W	2.0	-	-	-	-

Note: Lentils were cultivated in the season 2019. In the field of Farmer 3, Fe and Mn toxicities symptoms were visible in lentils. P and K were applied as triple superphosphate and potassium sulfate, respectively, before the sowing and incorporated into the soil through tillage. Farmer 3 applied N as urea after Fe and Mn symptoms became visible on leaves. Before sowing, the seeds were disinfected using the chemical products Mancozeb and Carbendazyme (Anagran® Plus, Santiago, Chile) to prevent fungal infection. * The sowing and threshing dates correspond to the beginning of wintertime and summertime in the southern hemisphere, respectively. L/P/W = lentils/potato/wheat.

We chemically analyzed the soils and the grains from these sites (Tables 2 and 3). Soil samples were taken from the soil surface (0–30 cm), air-dried and sieved to pass 2 mm before chemical analyses. Soil chemical analyses were measured according to the methods proposed by Sadzawka et al. [28] for Chilean soils. Briefly, available P was extracted by 0.5 M $NaHCO_3$ at pH 8.5 and measured by colorimetry by the molybdenum blue method; exchangeable cations (Ca, K, Mg and Na) were extracted by 1M CH_3COONH_4 at pH 7.0 and measured by atomic absorption spectroscopy (AAS); soil organic matter (SOM) was analyzed with the Walkley and Black oxidation procedure [29]. Soil pH was measured in water (pH_w 1:2.5) and electrical conductivity (EC) in the soil saturation extract according to Sadzawka et al. [28]. Fe, Mn, Zn and Cu were extracted by DTPA solutions and measured by ASS according to the method proposed by Lindsay and Norvell [30]. Plant available B in soil was extracted with hot $CaCl_2$ 0.01 M and determined colorimetrically following the azomethine-H method. Similarly, lentil grains were chemically analyzed to determine mineral elemental composition. Grains were dried in an oven at 65 °C and milled. Subsequently, the material was digested with HNO_3 and K, Ca, Mg, Cu, Zn, B, Mn and Fe were measured by AAS; total N was determined by an elemental analyzer (TruSpec Leco®, St. Joseph, MI, USA), and; P was measured by colorimetry by the vanadate-molybdate method.

2.2. Pot Experiment

We decided to carry out a pot experiment with the soil samples from plants which showed toxicity symptoms. Consequently, the pot experiment was conducted with the soil samples from the land of Farmer 3 (F3, Table 2), and they were collected specifically from those areas where the plants showed the more severe toxicity symptoms. The site has been cropped with a lentil-cereal-grassland rotation for at least 20 years around Chanco

(35°41′38.8″ S 72°30′10.6″ W). According to the Soil Taxonomy classification system the soil corresponds to a Mollisol (WRB/FAO system: Phaeozems) [31].

Table 2. Chemical soil analyses of the fields cultivated with lentils during the 2019–2020 season in the town of Chanco, Maule region, Central Chile.

	pH_w	EC	SOM	N	K	Ca	Mg	Na	Fe	P	Mn	Zn	Cu	B
		dS/m	g 100 g^{-1}			$cmol_{(+)}$ kg^{-1}			mg kg^{-1}					
F1, F2	5.7	0.14	4.8	11.8	0.92	7.87	2.73	0.14	100.1	15.9	16.7	3.42	1.24	0.74
F3	5.7	0.06	3.5	13.0	0.57	8.26	1.84	0.14	134.3	34.0	16.2	3.09	1.94	0.29
F4	5.8	0.05	1.8	1.0	0.18	5.82	3.14	0.20	90.2	11.0	19.0	1.14	1.34	0.12

Note: F = Farmer; EC = Electrical Conductivity; SOM = Soil Organic Matter. The extensive experiments designed as F1 and F2 were in the same fields and soils.

Table 3. Nutrient concentration of lentil grains cultivated in Chanco town, Maule region, central Chile. The *p*-value was obtained after ANOVA test. The small letters indicate statistical differences among sites according to Tukey test ($p < 0.05$) and SD is presented in parentheses.

Sites	N	P	K	Ca	Mg	B	Cu	Zn	Mn	Fe
			g 100 g^{-1}					mg kg^{-1}		
F1	3.77 (0.19)	0.31 (0.04) b	0.77 (0.04)	0.06 (0.02) b	0.11 (0.01)	5.75 (0.96)	6.75 (0.96)	41.00 (4.97)	15.50 (2.65) b	49.25 (5.56) b
F2	3.86 (0.48)	0.34 (0.10) b	0.82 (0.10)	0.05 (0.03) b	0.12 (0.02)	4.75 (1.26)	8.25 (1.26)	45.50 (9.57)	14.00 (0.82) b	53.50 (5.45) b
F3	3.86 (0.08)	0.48 (0.05) a	0.86 (0.15)	0.07 (0.01) ab	0.13 (0.01)	13.25 (10.97)	6.75 (0.96)	47.00 (3.92)	29.75 (6.24) a	97.25 (10.28) a
F4	3.64 (0.07)	0.30 (0.02) b	0.69 (0.01)	0.10 (0.02) a	0.11 (0.01)	5.75 (1.50)	7.50 (0.58)	44.25 (0.58)	16.50 (1.73) b	57.75 (4.11) b
p-Value	0.6	<0.01	0.1	<0.05	0.1	0.1	0.1	0.5	<0.001	<0.001

Note: F = Farmer.

The pot experiment was performed with 2.2 kg of soil in 2 L pots with six lentil plants grown during 157 days under greenhouse conditions. The treatments evaluated were three: (a) control (soil as in the field), (b) addition of lime ($CaCO_3$); and (c) addition of N (KNO_3). Each treatment consisted of six replicates. In the case of lime ($CaCO_3$) addition, the soil was incubated before the sowing for three weeks until it reached a pH_w of 6.6. We used analytical grade $CaCO_3$ with a purity of 99% (Merck®, Darmstadt, Germany). For the N treatment, 1 g N was partitioned during the growing period and added together with the irrigation as KNO_3. Basal P fertilization was applied for all treatments (50 mg P kg^{-1} of soil). All plants were inoculated with 2 mL per plant with a suspension of *Rhizobium leguminosarum* (RizoFix Gel®, BIOGRAM, Santiago, Chile) at rates of 1×10^9 cfu/mL to promote nodule formation. Pots were irrigated with demineralized water during the experiment to maintain a water content of 75% water-holding capacity. Demineralized water was used to prevent the addition of any other metal present in tap water that could contaminate the soil.

Shoots were collected at 103 days after sowing (DAS) (first harvest) and at the end of the experiment (157 DAS, second harvest), and they were dried at 65 °C until constant weight. Furthermore, in the first harvest, root length was determined by the procedure proposed by Tennant [32]. At the final harvest, shoot, root and grains were collected, dried and milled. Subsequently, plant material was analyzed using a portable energy-dispersive X-ray fluorescence (EDXRF) analyzer (Bruker AXS Inc., Billerica, MA, USA) to determine Fe and Mn concentrations. Ten samples of certified reference material from the Wageningen Evaluating Program for Analytical Laboratories [33] were used to obtain elements concentrations. This calibration consisted of converting K-alpha peak's photon counts into Fe and Mn concentrations (mg kg^{-1}) by least squares regression.

2.3. Data Analysis

Statistical analysis was performed using Statistica 9.0 (StatSoft, Inc., Tulsa, OK, USA). The pot experiment had a completely randomized design, with 3 treatments (n = 6). The addition of lime ($CaCO_3$) or N (KNO_3) were considered as main factors and subjected to one-way ANOVA [34]. In the case of yield and chemical composition of lentil grains collected from the field, the farmer was set as the main factor for the ANOVA analysis. The ANOVA test was followed by a Tukey test at the 5% level of significance to separate the means. Figures were drawn using GraphPad Prism® V8 (GraphPad Software, San Diego, CA, USA).

3. Results

Lentil grain yields cultivated under farmers' conditions ranged between 1.5 and 2.14 ton ha^{-1} (Figure 1).

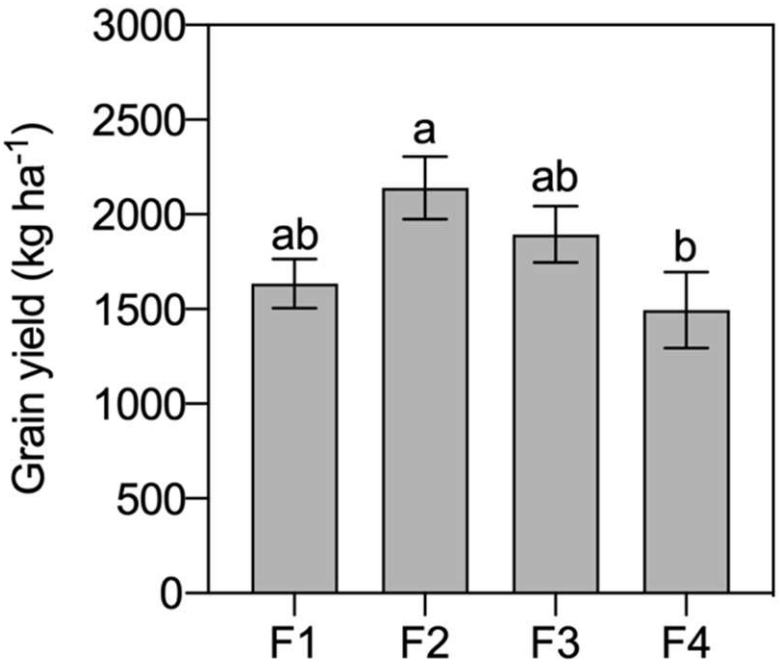

Figure 1. Grain yields in an extensive experimental site in Chanco, Maule region, central Chile. Small letters indicate differences among sites according to Tukey test ($p < 0.05$). F = farmer.

The site of more incidence of Fe and Mn toxicity was observed with Farmer 3. Soil Fe availability was higher in the soil of Farmer 3 while Mn availability was similar in all soils under lentil cultivation (Table 2). The mineral composition of lentil grains cultivated by Farmer 3 showed the highest nutrient concentrations among farmers' sites (Table 3). Fe and Mn in grains from Farmer 3 nearly doubled the concentrations obtained in grains of the other farmers, although this was not observed for Zn. Interestingly, B concentration was also higher for the grains cultivated by Farmer 3 than for the other farmers, but only in trends due to the high variability of the data (Table 3). Lentils grown in the farmer field with the highest Fe concentration showed toxicity symptoms with black and brown spots on leaves, chlorosis, and defoliation. However, the symptomatology was present in specific areas of this field of farmer 3 and this is why this did not mirror on grain yields, which was among the highest studied (Figure 1).

In the pot experiment, the dry matter (DM) produced by lentils in the intermediate harvest (103 DAS) was highest in the control plants (Figure 2A). The addition of either N or lime was not mirrored on DM increase in this intermediate harvest stage. Note that the DM is expressed in milligrams (mg) because of the low amount of biomass produced by

the plants at this stage. A slight reduction of roots was recorded for plants treated with N. At this stage, the amount of active and inactive nodules was counted. The plants treated with lime showed more nodules than those in the control, while almost no nodules were scored for those plants supplied with N (Figure 2B). The root to shoot (R/S) ratio indicates that the plants grown in the soil with the addition of lime developed relatively more roots compared to the other treatments and that N was not altering the extent of the root system at 103 DAS (Figure 2C).

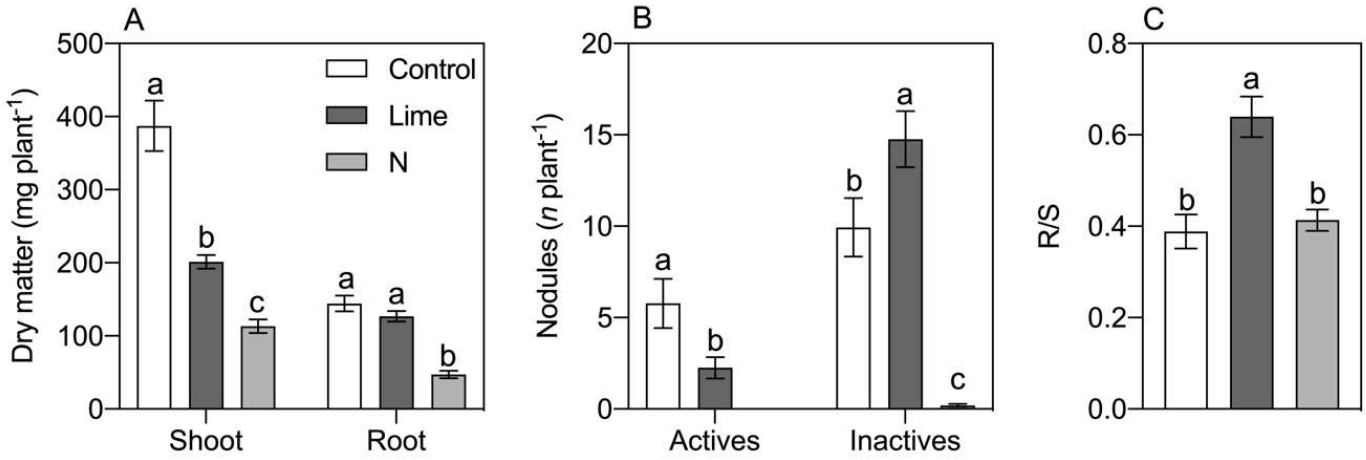

Figure 2. (**A**) Dry matter of shoots and roots produced by lentils grown in pots that were harvested 103 days after sowing, (**B**) number of active and inactive nodules, and (**C**) root to shoot ratio (R/S). Small letters indicate significant differences among treatments after Tukey's test ($p < 0.05$).

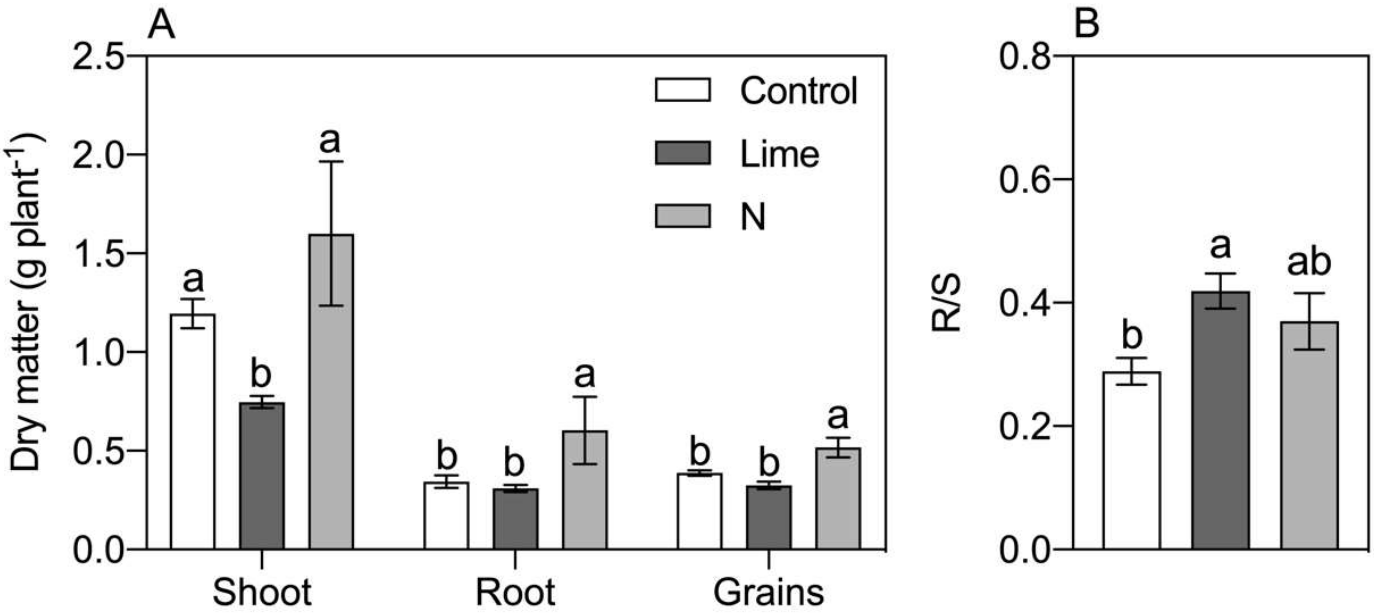

Figure 3. (**A**) Dry matter of shoots, roots and grains produced by lentils grown in pots that were harvested 157 days after sowing and (**B**) root to shoot ratio (R/S). Small letters indicate significant differences among treatments after Tukey's test ($p < 0.05$).

At the end of the experiment (157 DAS), the DM produced was higher in the plants that received N while the lowest DM production was recorded in those plants treated with lime (Figure 3A). Plants grown in the control soil and the soil treated with lime developed lower root DM than those plants grown with N addition. The grain yield also was positively affected by the addition of N being 33% higher than in the control plants. In the case of

plants grown in the soil with lime addition, the yield was almost not altered and was even slightly reduced in comparison to the yield obtained by the control plants. However, lime addition effectively diminished the number of spotted grains while the addition of N contributed to a significant reduction of spotted grains down to 12.5% while control presented about 37% of spotted grains (Figure 4). At the harvest time, Fe concentrations varied between 550 and 1200 mg kg^{-1} in shoots while Mn concentrations oscillated between 65 and 220 mg kg^{-1} (Figure 5A). In those plants fertilized with N, the Fe and Mn levels in the shoots were lower than in the control plants and those grown in soils treated with lime. In the case of Fe, it was significantly reduced to 50.5% in the plants treated with N relative to the control while the reduction of Mn was significant for plants grown in soils treated with either lime or with N (Figure 5A, bar graph). The reduction of Mn in the plants fertilized with N and lime corresponded roughly to 70% and 32.5%, respectively (Figure 5A, bar graph), compared to the control treatment. Roots exhibited high concentrations of Fe and Mn that were 13- and 9-fold higher than in the shoots, respectively (Figure 5B). In the roots of those plants fertilized with N, the Fe and Mn concentrations were significantly higher than in the other two treatments, showing an increase of around 27% compared to the control plants. The increase of Mn concentration in the roots of plants treated with N was also significantly different relative to the concentration of the control plants. In the plants grown in the soil treated with lime, the Fe and Mn concentrations of roots did not differ from those encountered in the control plants. In the grains, Fe concentration was reduced in plants treated with lime and N although only as a trend. However, a significant decrease was recorded on Mn concentration in the grains of plants treated with N. In general, Fe and Mn concentrations in grains were lower than in shoots and roots.

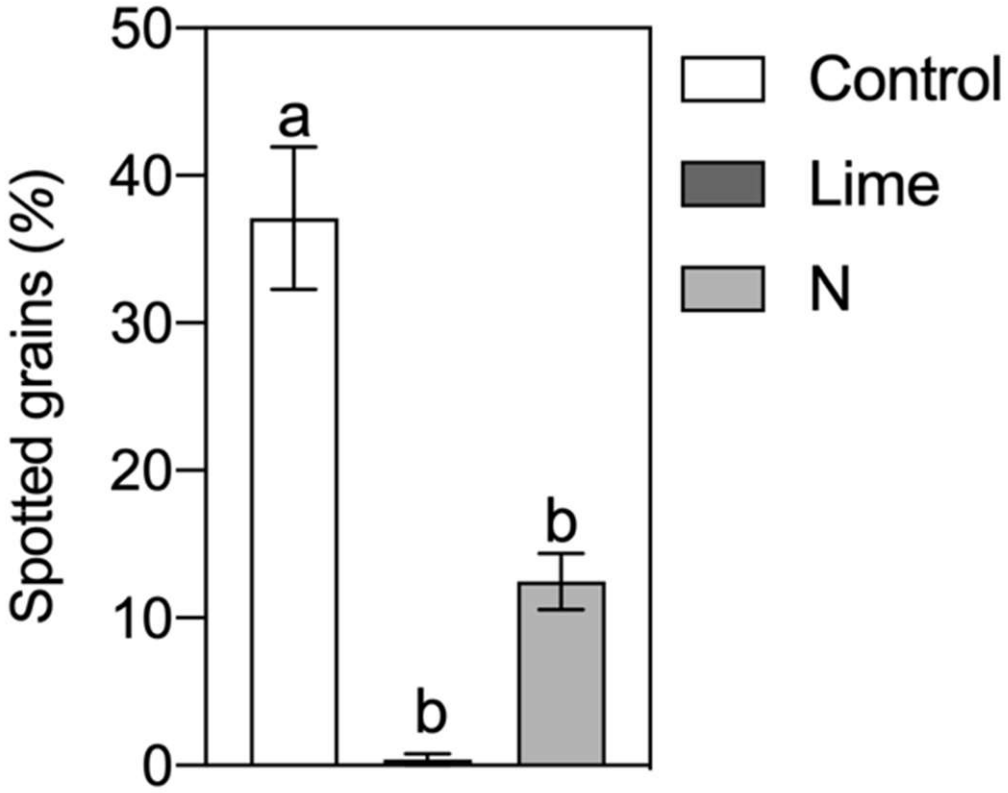

Figure 4. Percentage of spotted grains evaluated in lentils grown in pots depending upon the treatments with no nutrient addition, and lime and N applications. The percentage was calculated in relation to the total number of grains obtained in the respective treatment. Small letters indicate significant differences among treatments after Tukey's test ($p < 0.05$).

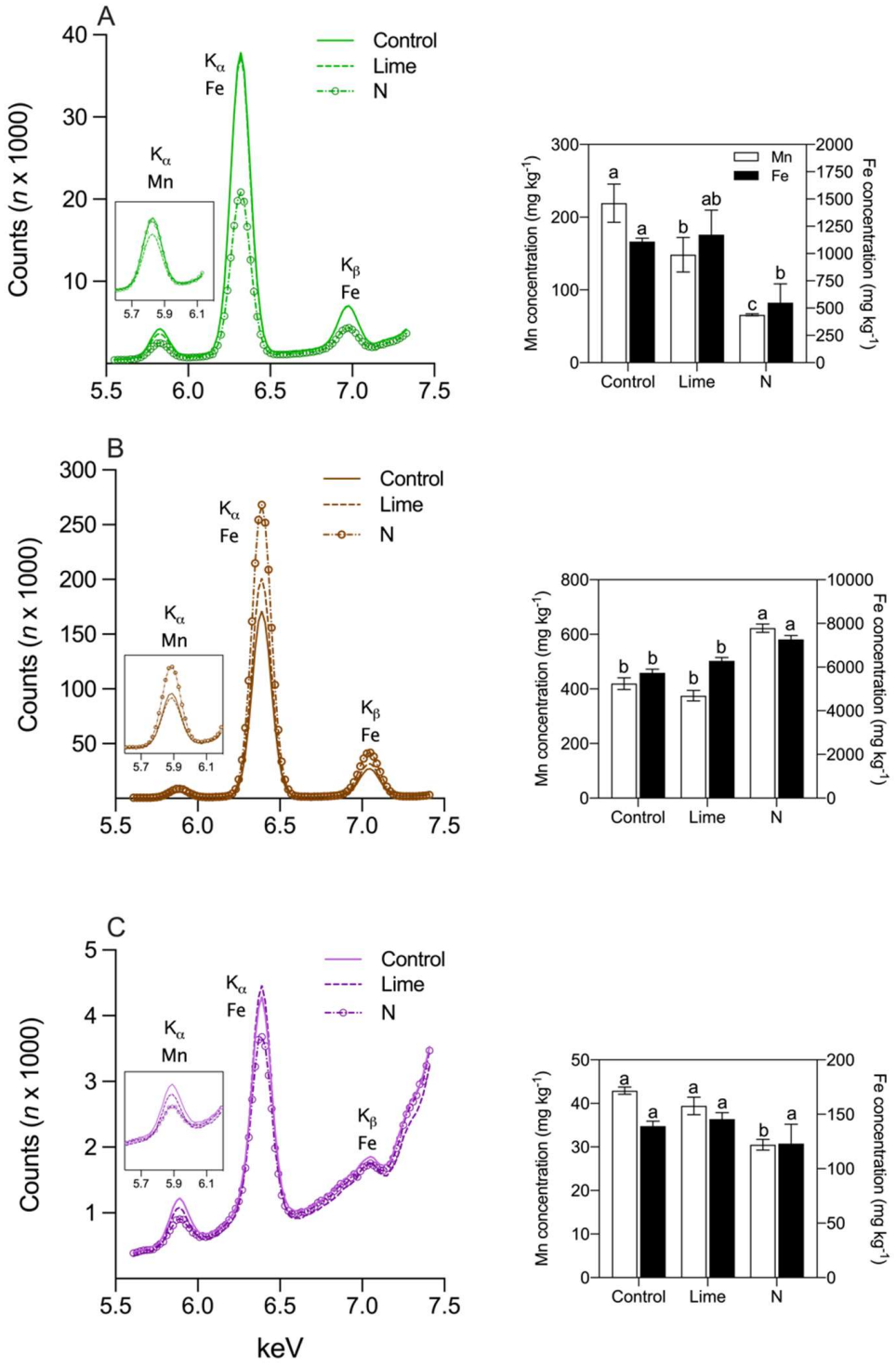

Figure 5. Fe and Mn concentrations in lentils grown in pots after 157 days after sowing. Curves show the X-ray fluorescence (XRF) spectra for Fe (K_α and K_β) and Mn (K_α) in: (**A**) shoots; (**B**) roots, and (**C**) grains. The bar graphs beside the curves indicate the calculated concentrations of Fe and Mn derived from the fluorescence spectra for each plant organ. Small letters in the concentration graphs indicate significant differences among treatments after Tukey's test ($p < 0.05$).

Control plants suffered Fe and Mn toxicities showing brown and black spots in basal leaves, and this symptomatology progressed until the complete fall of leaves (Figure 6A,B). The grains of control plants developed brown spots in the cuticle, and the grains looked sucked in some cases (Figure 6C, below).

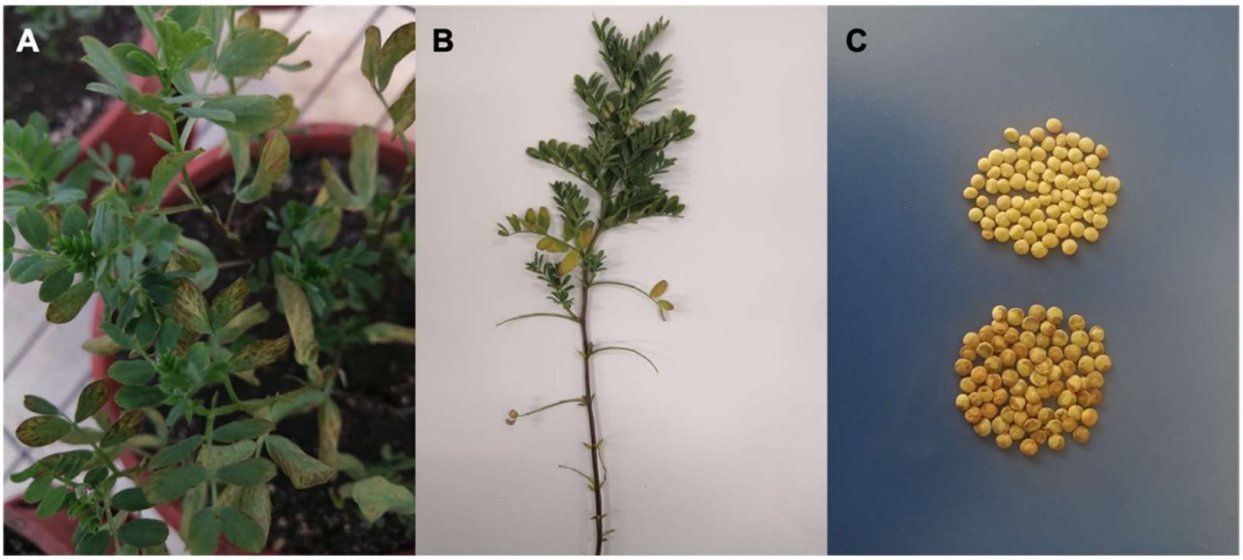

Figure 6. Lentil plants grown in pots with toxicity symptoms of Fe and Mn toxicities. (**A**) Brown and black spots in basal leaves and normal young leaves at the tip without symptomatology. (**B**) Complete defoliation of basal leaves and chlorotic progression in remanent leaves showing symptomatology of Fe and Mn toxicities. (**C**) Normal healthy lentil seeds (above) and spotted seeds with high Fe and Mn concentrations (below).

4. Discussion

Lentil production is negatively affected in Mediterranean areas where alkaline and calcareous soil conditions severely limit the availability of micronutrients [35,36]. In Chile, the lentil production area is concentrated in soils that vary in pH from slightly alkaline to slightly acidic conditions, although the main production zone is located where the soils have pH values below 7 [37]. For this reason, lentils grown in the Maule region rarely show deficiency symptoms associated with low Fe and Mn availability. However, soil properties in combination with unfavorable weather events trigger anaerobic soil conditions that promote high solubility of metals. This is related to poor aeration conditions caused by soil compaction or heavy textures [38]. Soil pH and redox potential are the main factors affecting the metal solubility in soils, although the redox potential plays a major role when the pH is in a narrow range or at a constant value, especially for Fe [14,18]. Similar effects due to reduction conditions were reported in a Chilean soil in which lentils showed symptoms of Fe and Mn toxicity [16]. In this soil, the change to a more negative redox potential increased by several fold the solubility of Fe and Mn, suggesting that the reductive conditions are likely responsible for the Fe and Mn toxicity levels found in lentils under field conditions. We collected soil samples from fields after harvest and after the rainfall season (Table 2), thus it is probable that the soil was aerated explaining the normal levels of Fe and Mn soil concentrations. In the field of Farmer 3, grains with toxicity symptoms attributable to Fe and Mn concentrations were analyzed. In this case, the grain yield was amongst the highest (Figure 1), but the commercial quality of the grains was negatively affected. The concentration of both elements in the grains of the field of Farmer 3 almost doubled the concentrations of the grains from the other farmers. In comparison, normal Fe and Mn concentrations of lentil grains range from 75.6 to 100 and 12.2 to 14.8 mg kg^{-1} [39], respectively, whose values were obtained from lentils cultivated in different sites in southern Saskatchewan, Canada. Although Fe concentration numbers for lentil grains from Farmer 3 is in the normal range, grain Mn concentration is almost double than the mean concentration reported by Ray et al. [39], which could explain the toxicity symptoms observed in the field of Farmer 3.

The DM produced by plants was increased by the addition of N but not by the addition of lime (Figure 3). Nodules in the plants treated with N were almost abolished while the amount of nodule in plants treated with lime increased. The inhibition of nodule formation

and N_2 fixation activity has been described by several authors [40–42]. The inhibition of nodule formation and the cessation of N_2 fixation is normally found in legumes fertilized with N since N uptake is energetically favored in comparison to the more expensive N_2 fixation process [43]. Although initially, the R/S ratio was lower for plants treated with N, it increased at the end of the growth period and it was higher relative to the other two treatments (i.e., control and lime treatments), indicating that a more extended root system was developed in the N treatment. The same was observable for the shoot DM and the grain yield attained. The increase of shoot DM could help to dilute the Fe and Mn concentrations absorbed by the plants, and at the same time, the more extended root system could contribute to store Fe and Mn (Figures 3 and 5). The high levels of Mn have been also associated to indole acetic acid (IAA) degradation in other species, which can impact plant development [44]. In fact, control plants were smaller than those grown with N addition.

Since unspecialized organs in lentils have been characterized so far for metal accumulation, as in other species, roots can help to store the excess of metals absorbed by the plant and buffer the Fe and Mn concentrations of the shoot. In case of overload of Fe due to unfavorable soil conditions, plants can store the Fe excess in shoots and roots by the synthesis of phytoferritin, which is localized in the cell plastids that store Fe and reduce the oxidative stress [45]. In other plant species, metal accumulation occurs mainly in specialized cell compartments or around organs such as in the base of trichomes in sunflower (*Helianthus annuus* L.), which is known to be a species resistant to Mn toxicity [46]. In addition, it has been described a transient accumulation of Mn in the roots of white lupins (*Lupinus albus*), which is rapidly mobilized to the shoots via xylem and subsequently stored in the shoots [47]. In lentils, Mn and Fe accumulation in the shoot resulted in dark spots in basal leaves, progressing to chlorotic and eventually turning them necrotic until complete defoliation (Figure 6), which agrees with the observations made by Horst [48] in cowpea (*Vigna unguiculata*). In cowpea leaves, the excess of Mn is visualized as brown or black spots as the result of higher Mn concentration in these areas [49]. Thus, it is likely that high Mn and/or Fe concentrations in lentils are localized in the vacuoles of leaves' cells as has been observed for common beans [50]. More recently, vacuolar metal transporters have been indicated to play a central role in the homeostasis of Mn in plants, either by sequestering the excess of this metal or by retrieving it from the vacuole when deficiency emerges [51,52]. The N_2 fixation activity by rhizobia can reduce the pH values in the rhizosphere and thereby increases the solubility of micronutrients [26,53]. It seems likely that legumes that depend solely on N_2 fixation are more sensitive to high Mn availability that affects plant growth, nodule number and N_2 fixation [54,55]. This is probably connected with the fine regulation of nodule activity and N demand, which results in a reduction of N_2 fixation when a stress emerges and it disturbs normal plant growth [56].

To reduce the incidence of this mineral disorder, lentils should be sown in well-drained soils and be fertilized with moderate N doses at the sowing time to ensure the establishment of the plants, or by adjusting the sowing time after the rainy season. For the latter, it is necessary to advance in breeding programs to produce short-cycle lentil varieties that can achieve high yields in shorter growing periods. Progress must also be made in understanding the genetic and the molecular mechanisms of Fe and Mn transport and their storage in roots and leaves of lentils, which could open new paths for increasing micronutrient concentrations of seeds and for increasing their yields.

5. Conclusions

Lentils that rely solely on N_2 fixation for their N nutrition are more likely to suffer Fe and Mn toxicity, thus this works highlights the importance of using N fertilization to increase the DM production of lentils. This would reduce Fe and Mn concentrations on leaves to a level that is possibly under the threshold that causes toxicity in plant tissues. Furthermore, the increase of Fe and Mn in the roots is likely connected to the reduction of these metals on the shoots. Since reduction conditions are more prevalent during the

lentil growing season in the type of soil studied in this work, the lime addition to augment soil pH value has little effect if soil is not managed to increase its aeration. Finally, further research is required on Fe and Mn compartmentalization in different lentil organs, which could help us to understand how this crop deals with metal toxicities, allowing progress towards understanding the physiological and the molecular controls of metal transport in this species.

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