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Enhancing alfalfa productivity and nutritional quality through zinc biofortification with conventional and nano zinc oxide

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ABSTRACT

Climate change and pervasive zinc deficiency have increased hidden hunger by decreasing the nutritional quality of cereal and forage crops. Zinc biofortification offers a promising and sustainable technique to enhance crop zinc concentration and support livestock nutrition. The present research enhanced alfalfa yield, quality and zinc content by implementing zinc sources. The experiment was performed during the Rabi season around mid-October. Two cultivars of alfalfa, V_1 =Supersonic FD 9 (Australian Origin) and V2 = Sargodha Lucerne 2002 (Pakistan Origin), were employed in the experiment. The experiment consists of two sources of zinc, S_1 = $ZnSO_4$ and S_2 = Zinc oxide nanoparticles, with four different zinc doses: L1 = 0 kg/ha, L2 = 3 kg/ha, L3 = 6 kg/ha, L4 = 9 kg/ha. Results revealed that nano ZnO markedly enhanced alfalfa performance compared with conventional ZnO. The highest shoot fresh weight (26.57 g), root fresh weight (2.10 g), number of leaves (76.0 plant^{-1}), chlorophyll *a* (1.690 mg g^{-1}), chlorophyll *b* (2.356 mg g^{-1}), total chlorophyll (3.980 mg g^{-1}), crude protein (24.48%), crude fiber (29.48%), green fodder yield (49,626.67 kg ha^{-1}), dry matter yield (9,925.33 kg ha^{-1}), and zinc content (84.53 mg kg^{-1}) were obtained in variety V2 under nano ZnO at the highest zinc level, representing the best overall treatment performance.

Keywords: alfalfa, zinc biofortification, conventional zinc oxide, nano zinc oxide, forage quality.

INTRODUCTION

The most popular perennial fodder legume in the world, alfalfa (*Medicago sativa* L.), belongs to the Fabaceae family (Feier et al., 2023). Owing to its highest nutrient contents and dry matter yield, alfalfa is substantial for forage production around the world. Alfalfa belongs to the Mediterranean basin, although extensively scattered around Europe (Hadidi et al., 2023). Alfalfa is widely recognized as the "Queen of Forages" because of its high biomass production, excellent nutritional quality, and the ability to adjust to a variety of environmental conditions (Feier et al., 2023). It is an excellent fodder legume composes huge levels of crude proteins and secondary metabolites. Alfalfa minimizes greenhouse gas emissions from fertilizers, maintains water via good soil structure and boosts biodiversity (Naseer et al., 2026; Shakeel et al., 2021)

Malnutrition, sometimes termed as hidden hunger widespread in the world. Multiple developing countries are dealing with a silent raise in the shortage of micronutrients in fodders. (Sajjad et al., 2026). Due to the lack of micronutrients, the level of vital nutrients also decreased, which affects animal health. It relates to insufficient intake, absorption and utilization of essential minerals. (Campisi et al., 2021). It performs an irreplaceable role in biochemical processes comprising metabolic reactions, immune responses, and synthesis of various enzymes and proteins (Rashid et al., 2023). An animal demands 20-75 mg kg^{-1} of zinc, although deficiency causes anorexia, reduced growth, impaired health and bone tissues, increased susceptibility to infection, and skin parakeratosis (Ranjan et al., 2021). Zinc deficiency is commonly claimed in ruminants, particularly in regions with zinc-deficient soils. It is substantial for completing worldwide food security and improved health outcomes by managing zinc deficiencies with dietary amendment, biofortification, and agricultural modifications (Amin et al., 2023).

Biofortification, a potent biological technique, is one possibility to reduce micronutrient inadequacy. It is a beneficial tool in resisting hunger and micronutrient starvation (Windsor et al., 2024). The livestock sector has emerged as the biggest contributor to agriculture, accounting for almost 62.68% of agriculture and 14.36% of the national GDP. Livestock depend on grains and fodder for feed, which could be low in nutrients due to changes in climate. This method enhances the nutritional worth of crops by means of agronomic biofortification, including the application of zinc fertilizer, seed priming, genetic practices, and microbial biofortification (Kiran et al., 2022). ZnONPs support crop growth and improve soil health, presenting a controlled approach to zinc applications in agriculture (Kiani et al., 2024).

Despite the growing concern in zinc biofortification, restricted information is available on the comparative effectiveness of conventional zinc oxide and zinc oxide nanoparticles in improving zinc accumulation and fodder quality in alfalfa. Furthermore, the potential of ZnO nanoparticles as an innovative approach for improving zinc bioavailability in alfalfa remains largely unexplored. Although both conventional $ZnSO_4$ and nano-ZnO have been investigated as zinc sources, limited information is available on their comparative effectiveness for improving zinc accumulation, growth, yield, and nutritional quality of alfalfa under the same experimental conditions. Therefore, the present study was designed to directly compare conventional $ZnSO_4$ with nano-ZnO at different Zn application levels, providing insights into their relative effectiveness for alfalfa biofortification and nutritional improvement. The implementation of an optimal zinc source will enhance zinc biofortification while increasing overall crop performance. This research aimed to evaluate the capability of soil-applied zinc sources in improving zinc accumulation and fodder quality, and to recognize the most effective zinc source for zinc biofortification and enhanced forage productivity.

MATERIALS AND METHODS

Experimental site and design

At the Student Research Farm, Department of Agronomy, University of Agriculture Faisalabad, at elevation of 184 meters above sea level, field research was carried out to examine zinc biofortification for improving forage yield and nutritional quality in alfalfa (*Medicago sativa* L.). This area falls under a semi-arid subtropical climate zone, geographically lying at 31°26′ N latitude and 73°06′ E longitude. The experiment was carried out in a randomized complete block design using a split-split plot arrangement with three replications. The experiment was performed during the Rabi season around mid-October. Two cultivars of alfalfa, V_1 =Supersonic FD 9 (Australian Origin) and V2 = Sargodha Lucerne 2002 (Pakistan Origin), were employed in the experiment. The experiment consists of two sources of zinc, S_1 = $ZnSO_4$ and S_2 = Zinc oxide nanoparticles, with four different zinc doses: L1 = 0 kg/ha, L2 = 3 kg/ha, L3 = 6 kg/ha, L4 = 9 kg/ha. Treatments were subsequently applied 30 and 45 days after sowing. Data recorded after 15 days of the 2nd application. Table 1 displays the physiochemical characteristics of the experimental soil. Additionally, the University of Agriculture Faisalabad's Meteorological Cell provided the meteorological data used in this study, which is shown in Figure 1.

Crop husbandry

Recommended N, P, and K fertilizers were applied uniformly to all treatments in addition to the zinc treatments. This ensured adequate plant nutrition and allowed the effects of zinc application to be evaluated accurately. The nano-ZnO used in this study was commercially procured, and its physicochemical characteristics were based on the specifications provided by the manufacturer. The product information, including particle size, purity, and other available characteristics, has been added to the Materials and Methods section to improve the reproducibility of the study. Each experimental plot measured 1.5 × 1.5 m (2.25 m^2). Alfalfa was sown at a row spacing of 20 cm using a seed rate of 20 kg ha^{-1}. Recommended N, P, and K fertilizers were applied uniformly to all treatments at 23, 35, and 40 kg ha^{-1}, respectively. Zinc treatments ($ZnSO_4$ and nano-ZnO) were applied to the soil at 30 and 45 days after sowing at the specified rates of 0, 3, 6, and 9 kg ha^{-1}. The required quantity of each zinc source was weighed according to the treatment rate, mixed thoroughly with soil, and uniformly applied around the plants to ensure even distribution. All other agronomic practices were kept uniform across treatments.

Data recording procedure

Growth attributes

The plants were properly removed and thoroughly cleaned with water. Roots were separated by means of a blade, and the fresh weight was measured in grams by using a weight balance. Plants were uprooted and cleaned with water. Roots were detached, and the fresh weight was determined through a weight balance. Each plant's number of leaves was counted and noted.

Biochemical attributes

Chlorophyll pigments were calculated by the protocol of Arnon (1949). In 80% acetone, fresh plant leaves were chopped and placed in the refrigerator overnight. The spectrophotometer concentrations of total chlorophyll, chlorophyll a, and chlorophyll b were recorded at 665, 663, and 480 nm. Given formula used to evaluate the chlorophyll contents.

$$\text{Chlorophyll a (fresh leaf weight mg/g)} = [12.7(OD663) - 2.69(OD645)] \times \frac{V}{1000} \times W \quad (1)$$

$$\text{Chlorophyll b (fresh leaf weight mg/g)} = [22.9(OD645) - 4.68(OD663)] \times \frac{V}{1000} \times W \quad (2)$$

$$\text{Total Chlorophyll} = \text{Chlorophyll } a + \text{chlorophyll } b \quad (3)$$

where: V – Volume of acetone in extract (mL) and W – Weight of fresh leaf (mg).

Quality attributes

The Kjeldahl technique was used to calculate the crude protein content. A 5 g digesting mixture and 30 mL of concentrated H_2SO_4 were used to digest one gram of oven-dried material. Distilled water was used to dilute the digest to 250 mL. 10 mL of 40% NaOH was used to distill a 10 mL aliquot, and the ammonia that was liberated was collected in 10 mL of 4% boric acid that included methyl red indicator. Using 0.1 N H_2SO_4, the trapped ammonia was titrated to a light-pink endpoint. To estimate the amount of crude protein, the nitrogen content was determined by multiplying the amount of acid consumed by 6.25 (Goyal et al., 2022). The Soxhlet and acid-alkali digestion methods were used to calculate the crude fiber content. Two grams of oven-dried sample were defatted using a Soxhlet device, followed by sequential digestion with 1.25% H_2SO_4 and 1.25% NaOH for 30 min each. The residue was cleaned, dried to constant weight at 105°C, then ashed at 600

Table 1. Soil physiochemical attributes

Characteristics	Phosphorus	Potassium	Nitrogen	Saturation	Electrical conductivity	Organic matter	Texture	pH
Unit	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	%	dSm^{-1}	%	-	-
Values	33.4	162	150	36	1.08	1.62	Loam	7.2

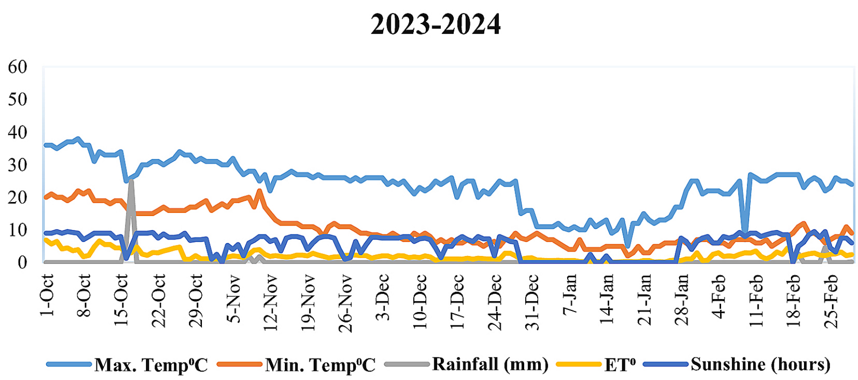

Figure 1. The experimental site's daily minimum and maximum temperatures, rainfall, ET, and relative humidity throughout the growth seasons

°C in a muffle furnace. The dry residue and ash weights were subtracted to determine the crude fiber content. A cotton-sealed extraction thimble containing two grams of dried material. The Soxhlet extraction equipment was filled with the thimble. A condenser was attached to a 250 mL flask containing ether. Ether evaporated at 40–60 °C, and vapors condensed. Following the extraction of fat, the sample was aligned in the flask using the siphon tube. It took six to eight hours to extract all of the fat. Ether evaporated and fat shifted in petri dish and was calculated by using the formula.

Yield attributes

After harvesting, fresh forage yield was estimated by an electronic scale. From each plot, chopped fodder was dried in a laboratory oven until the sample acquired a constant weight. An electronic balance was used to calculate the dry weight.

Total zinc content

After harvesting, total zinc content was evaluated through plant dry matter (Elango et al., 2021).

Statistical analysis

Fisher's analysis of variance (ANOVA) was used to evaluate the data, and Tukey's honestly significant difference (HSD) test was used to compare treatment means at the 5% probability level (Steel and Torrie, 1997).

RESULTS

Statistical analysis of the studied parameters presented in Table 2.

Growth attributes

Shoot fresh weight (SFW)

Regardless of the zinc source, shoot fresh weight increased steadily with increasing zinc levels from L1 to L4 in both varieties. Data presented in Figure 2a. Shoot fresh weight significantly increased with increasing zinc levels, with nano-ZnO producing a greater response than $ZnSO_4$. Variety V2 also showed significantly higher shoot fresh weight than V1, with the highest response observed at V2 × S2 × L4. At each zinc level, S2 resulted in greater shoot fresh weight than S1, and V2 consistently exhibited higher values than V1. The highest shoot fresh weight (26.57 g) was obtained from the combination of V2 × S2 × L4, followed by V1 × S2 × L4 (23.53 g), whereas the lowest value (14.00 g) was recorded in V1 × S1 × L1.

Root fresh weight (RFW)

Results presented in Figure 2b as under S1, root fresh weight in V1 increased from 0.71 g at L1 to 1.16 g at L4, whereas V2 recorded values ranging from 0.74 g at L1 to 1.60 g at L4. A similar increasing trend was observed under S2, where V1 produced 0.78, 0.88, 0.96, and 1.63 g at L1, L2, L3, and L4, respectively, while V2 recorded 0.83, 0.95, 1.03, and 2.10 g at the corresponding zinc levels. Comparison between zinc sources revealed that S2 consistently produced greater root fresh weight than S1 at each zinc level in both varieties. Similarly, V2 had greater root fresh weight compared to V1 for all treatment combinations, especially at the highest concentration of zinc. The maximum root fresh weight (2.10 g) was recorded by V2 subjected to S2 at L4, and the second highest (1.63 g) by V1 subjected to S2 at L4, while the lowest (0.71 g) was recorded by V1 subjected to S1 at L1.

Number of leaves

As shown in Figure 2c for zinc source S1, variety V1 produced 22.33 leaves per plant at L1. This number increased to 33.00 leaves at L2, 36.67 leaves at L3, and reached 63.00 leaves at L4. In comparison, variety V2 had a higher leaf count at all zinc levels, recording 28.00, 40.00, 54.67, and 72.00 leaves per plant at L1, L2, L3, and L4, respectively. The same observation was made under zinc source S2, whereby variety V1 had 24.00 leaves per plant at L1, then 36.00 at L2, 50.33 at L3, and 64.00 at L4. Similarly, variety V2 had 30.00, 48.67, 59.33, and 76.00 leaves per plant at respective zinc levels. It is evident that variety V2 had more leaves compared to V1 under both zinc sources at each zinc level. It should also be noted that zinc source S2 stimulated more leaf formation compared to S1, especially at moderate and higher zinc levels. The maximum number of leaves (76.00 leaves plant^{-1}) was obtained from the treatment combination V2 × S2 × L4, whereas the minimum value (22.33 leaves plant^{-1}) was recorded in V1 × S1 × L1.

Table 2. F-values for the following parameters

Parameters	Variety	Zinc source	Zinc level
Shoot fresh weight	144.5**	23.12**	63.57**
Root fresh weight	3.74ns	24.34**	64.93**
Number of leaves	43.49*	9.81*	81.35**
Chlorophyll a	108.91**	35.74**	88.18**
Chlorophyll b	152.41**	16.42*	10.91**
Total chlorophyll	11.96ns	17.4*	12.28**
Crude protein	169.05**	290.91**	1184.52**
Crude fiber	3432.13**	12444**	9878.08**
Crude fat	3.44ns	2.51ns	42.52**
Green fodder yield	4609.97**	88.3**	3400.96**
Dry matter yield	4.61**	8.83**	3.40**
Zinc content	19.97*	75.53**	34682.2**

Biochemical attributes

Chlorophyll a

As shown in (Figure 3d) under zinc source S1, variety V1 recorded chlorophyll a values of 0.448 at L1, 0.835 at L2, 0.987 at L3, and 1.105 at L4. In comparison, variety V2 consistently exhibited higher chlorophyll a contents of 0.782, 0.959, 1.163, and 1.381 at L1, L2, L3, and L4, respectively. A similar increasing trend was observed under zinc source S2, where chlorophyll a content in V1 increased from 0.738 at L1 to 0.922 at L2, 1.107 at L3, and 1.227 at L4. Likewise, variety V2 recorded chlorophyll a values of 0.838, 0.976, 1.288, and 1.690 at the corresponding zinc levels. Comparison between the two varieties indicated that V2 maintained higher chlorophyll a content than V1 across all zinc levels under both zinc sources. Moreover, zinc source S2 generally resulted in greater chlorophyll a accumulation than S1, particularly at the higher zinc levels. The highest chlorophyll a content (1.690) was observed in the treatment combination V2 × S2 × L4, whereas the lowest value (0.448) was recorded in V1 × S1 × L1.

Chlorophyll b

Figure 3e demonstrates as chlorophyll b content exhibited a marked improvement in response to increasing zinc levels, although the magnitude of response varied between varieties and zinc sources. Under zinc source S1, chlorophyll b content in variety V1 increased from 0.726 at L1 to 0.981 at L2 and 1.174 at L3, reaching a maximum of 1.324 at L4. In variety V2, the corresponding values were 0.983, 1.282, 1.567, and 1.326 at L1, L2, L3, and L4, respectively, indicating an increase up to L3 followed by a slight decline at L4. Under zinc source S2, both varieties showed a continuous increase in chlorophyll b content with increasing zinc levels. Variety V1 recorded values of 0.777, 1.109, 1.326, and 1.625 at L1, L2, L3, and L4, respectively, while variety V2 produced substantially higher values of 1.310, 2.035, 2.268, and 2.356 across the respective zinc levels. Overall, variety V2 accumulated more chlorophyll b than V1 under both zinc sources, with the superiority of S2 becoming more evident at the higher zinc levels. The highest chlorophyll b content (2.356) was obtained from the treatment combination V2 × S2 × L4, whereas the lowest value (0.726) was recorded in V1 × S1 × L1.

Total chlorophyll

According to (Figure 3f) total chlorophyll content responded positively to zinc application in both varieties, with higher zinc levels generally promoting greater pigment accumulation. For zinc treatment S1, variety V1 showed chlorophyll content levels of 1.297 for L1, while these rose to 1.816 at L2, 2.160 at L3, and finally 2.428 at L4. For variety V2, the chlorophyll content level was observed to be 1.764 for L1, rising up to 2.240 at L2 and 2.729 at L3, followed by a slight reduction to 2.706 at L4. Under zinc source S2, a continuous increase in total chlorophyll content was observed across all zinc levels. Variety V1 exhibited values of 1.514, 2.031,

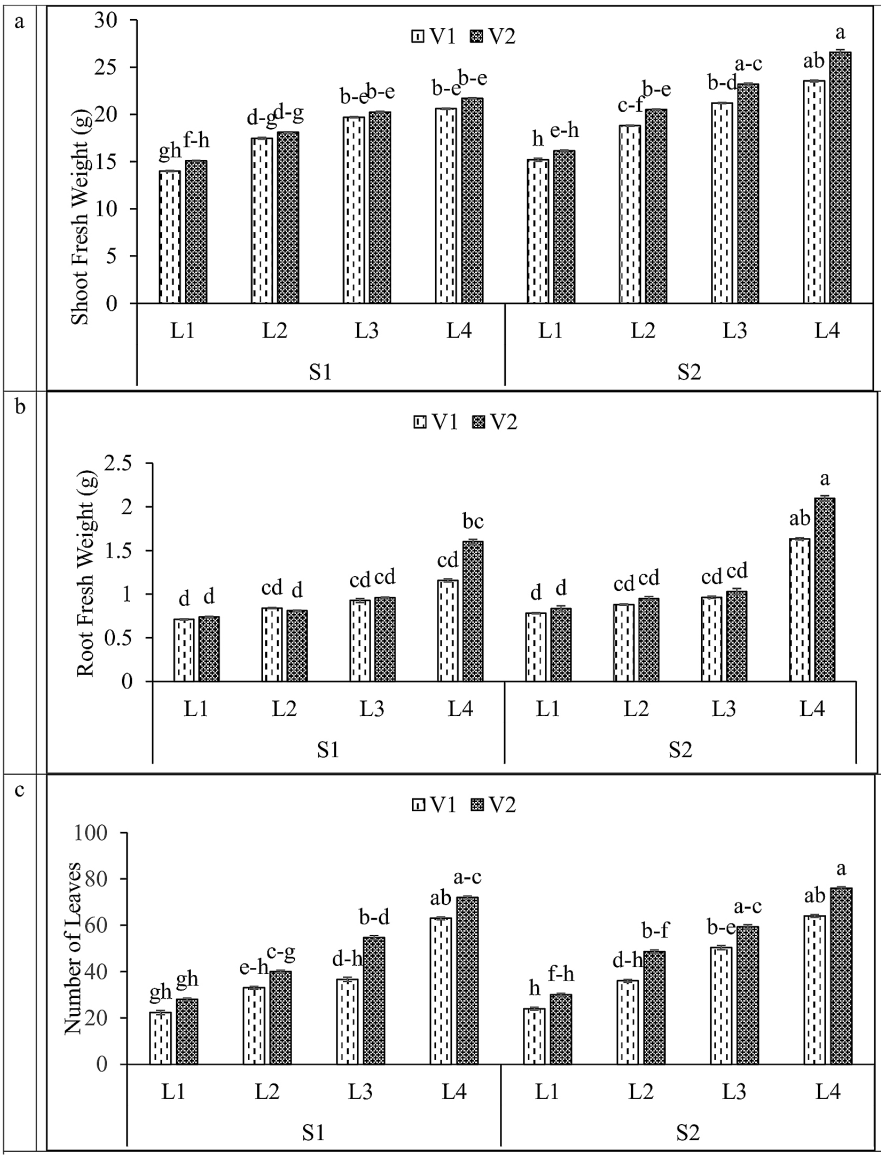

Figure 2. Effect of conventional and nano ZnO on shoot fresh weight, shoot dry weight and root fresh weight of alfalfa

V1 =Supersonic FD 9 (Australian Origin) and V2 = Sargodha Lucerne 2002 (Pakistan Origin), S1 = $ZnSO_4$ and S2 = Zinc oxide nanoparticles, with four different zinc doses: L1 = 0 kg/ha, L2 = 3 kg/ha, L3 = 6 kg/ha, L4 = 9 kg/ha Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$.

2.432, and 2.852 at L1, L2, L3, and L4, respectively, whereas variety V2 produced considerably higher values of 2.718, 3.409, 3.555, and 3.980 at the corresponding zinc levels. Across both zinc sources, variety V2 consistently maintained higher total chlorophyll content than variety V1, while zinc source S2 proved more effective than S1 in enhancing chlorophyll accumulation, especially at the higher zinc levels. The highest total chlorophyll content (3.980) was recorded in the treatment combination V2 × S2 × L4, whereas the lowest value (1.297) was observed in V1 × S1 × L1.

Quality attributes

Crude protein

Crude protein content increased gradually with increasing zinc levels in both varieties under both zinc sources. As depicted in (Figure 4g) under zinc source S1, variety V1 recorded crude protein contents of 18.35 at L1, which increased to 19.23 at L2, 20.32 at L3, and 21.19 at L4. Likewise, variety V2 exhibited a continuous increase from 19.23 at L1 to 20.32 at L2, 21.41 at L3, and reached 23.38 at L4. Under zinc source S2, crude protein content in variety V1 increased from 18.72 at L1 to 19.74

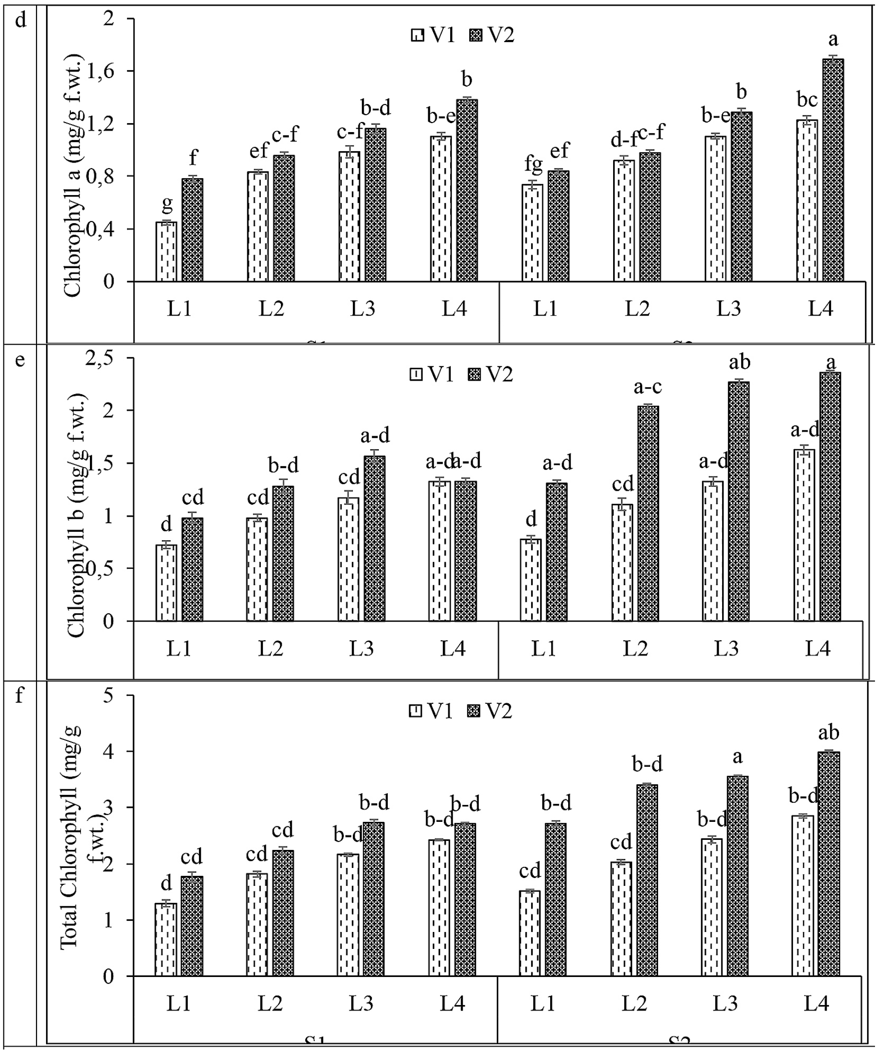

Figure 3. Effect of conventional and nano ZnO on chlorophyll a, chlorophyll b and total Chlorophyll of alfalfa. V_1 =Supersonic FD 9 (Australian Origin) and V2 = Sargodha Lucerne 2002 (Pakistan Origin), S_1 = $ZnSO_4$ and S_2 = Zinc oxide nanoparticles, with four different zinc doses: L1 = 0 kg/ha, L2 = 3 kg/ha, L3 = 6 kg/ha, L4 = 9 kg/ha Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$.

at L2, followed by 20.76 at L3 and 21.78 at L4. In variety V2 also, there was progressive increase in the content of crude protein from 19.88 at L1 to 20.97 at L2, 22.94 at L3, and 24.47 at L4. It was found out that variety V2 had always shown higher content of crude protein than variety V1 at all the three zinc levels irrespective of the zinc source used. Moreover, there was also more crude protein produced by the zinc source S2 than S1, but the differences were more at the higher levels of zinc. Highest percentage of crude protein (24.47%) was found in V2 × S2 × L4 while the minimum percentage (18.35) was in V1 × S1 × L1.

Crude fiber

The crude fiber was significantly affected by the variety, zinc sources, zinc levels, and interactions among them. As can be seen from Figure 4(h), the results showed an increasing trend in crude fiber with increasing levels of zinc in both varieties under both sources of zinc. In variety 1, the crude fiber for S1 increased from 16.29 at L1 to 24.95 at L4, whereas that of S2 increased from 18.37 to 26.27 at the same levels of zinc. Likewise, V2 recorded values of 19.32, 21.49, 24.49, and 26.61 under S1, whereas under S2 the corresponding values were 21.21, 24.62, 27.36, and 29.48. At each zinc level, S2 had higher crude fiber contents compared to S1 in both genotypes. In addition, V2 showed higher crude fiber content compared to V1 regardless of the zinc source and zinc levels. The maximum value of crude fiber (29.48) was obtained from the treatment combination V2 × S2 × L4, followed by V2 × S2 × L3 (27.36) and V1 × S2 × L4 (26.27), while the lowest value (16.29) was recorded in V1 × S1 × L1.

Crude fat

Crude fat content showed a significant difference due to the variety, zinc source, zinc rate, and their interactions. It can be seen from (Figure 4i), that the effect of zinc on the crude fat varied with the variety and zinc source, however, higher zinc rates always increased the crude fat. Under S1, crude fat in V1 increased from 2.29 at L1 to 2.97 at L4, while V2 recorded values of 2.45, 2.81, 2.76, and 3.34 across L1 to L4. Crude fat of V1 in S2 was 2.37, 2.67, 2.85, and 2.84 at L1, L2, L3, and L4, respectively, showing a mild decrease in the highest zinc concentration. In comparison to this, the crude fat for V2 was 2.58, 2.84, 2.12, and 3.31 in treatments S2, L1, L2, L3, and L4, respectively, showing a slight decrease in L3 followed by a remarkable increase in L4. In general, V2 had higher values of crude fat as compared to V1 in most cases, whereas S2 was better than S1. The highest crude fat value (3.34) was recorded in V2 × S1 × L4, closely followed by V2 × S2 × L4 (3.31), whereas the lowest value (2.12) was observed in V2 × S2 × L3. This observation shows that the effect of zinc on the formation of crude fats is based on the type of zinc used and variety, thereby causing a considerable interaction between the study factors.

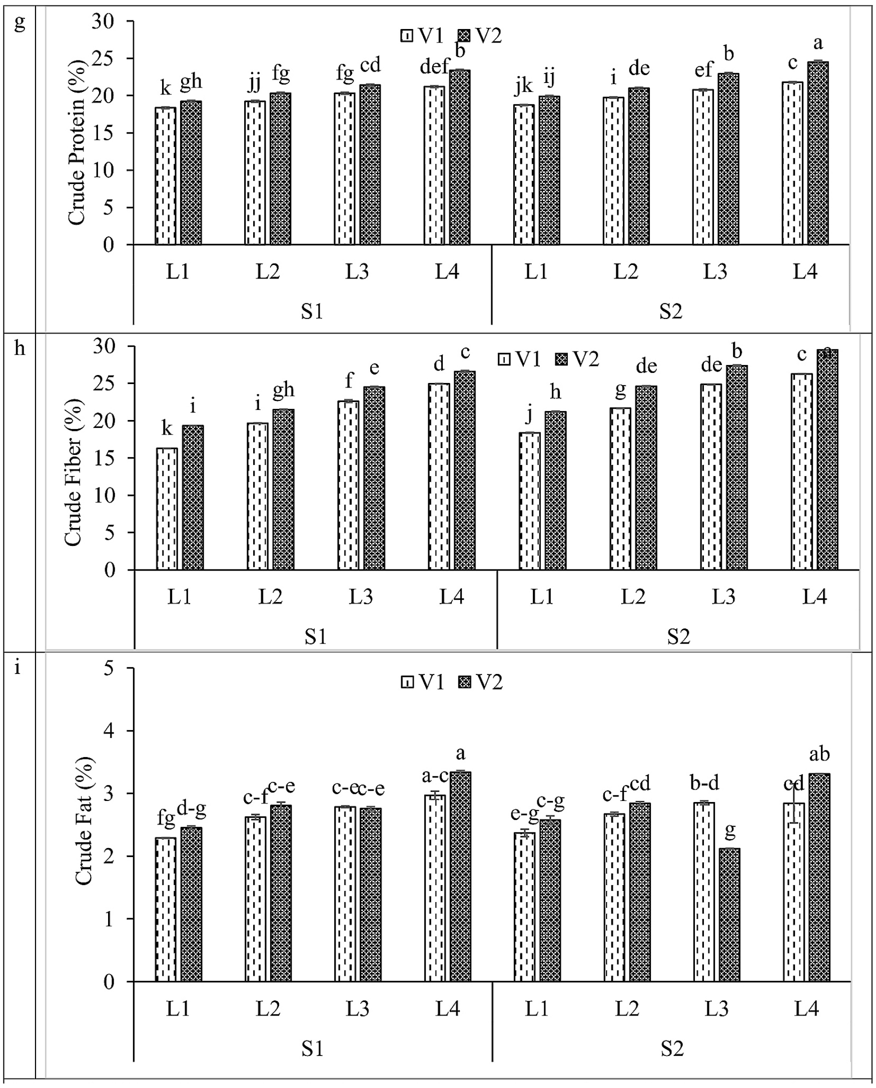

Figure 4. Effect of conventional and nano ZnO on crude protein, crude fiber and crude fat of alfalfa V_1 = Supersonic FD 9 (Australian Origin) and V2 = Sargodha Lucerne 2002 (Pakistan Origin), S_1 = $ZnSO_4$ and S_2 = Zinc oxide nanoparticles, with four different zinc doses: L1 = 0 kg/ha, L2 = 3 kg/ha, L3 = 6 kg/ha, L4 = 9 kg/ha. Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$

Yield attributes

Green fodder yield

Variety V1, shown in (Figure 5j) under zinc source S1, yielded 28,800 green fodder at L1, which rose to 35,666.67 at L2, 40,866.67 at L3, and 45,000 at L4. Similarly, variety V2 recorded higher green fodder yields of 33,613.33, 38,400, 43,426.67, and 46,666.67 at L1, L2, L3, and L4, respectively. Variety V1's green fodder yield rose under zinc source S2 from 32,066.67 at L1 to 36,333.33 at L2, then to 41,960 at L3 and 45,640 at L4. Likewise, variety V2 exhibited yields of 35,600, 40,933.33, 45,600, and 49,626.67 across the respective zinc levels. Comparison between the two varieties showed that variety V2 consistently produced greater green fodder yield than variety V1 under both zinc sources at all zinc levels. In addition, zinc source S2 generally resulted in higher green fodder yield than S1, with the improvement becoming more evident at the higher zinc application levels. The maximum green fodder yield (49,626.67) was obtained in the treatment combination V2 × S2 × L4, whereas the minimum yield (28,800) was recorded in V1 × S1 × L1.

Dry matter yield

There was an upward trend in the dry matter yield with successive increases in zinc level for both varieties under each zinc source. Depicted in (Figure 5k) under S1, V1 produced

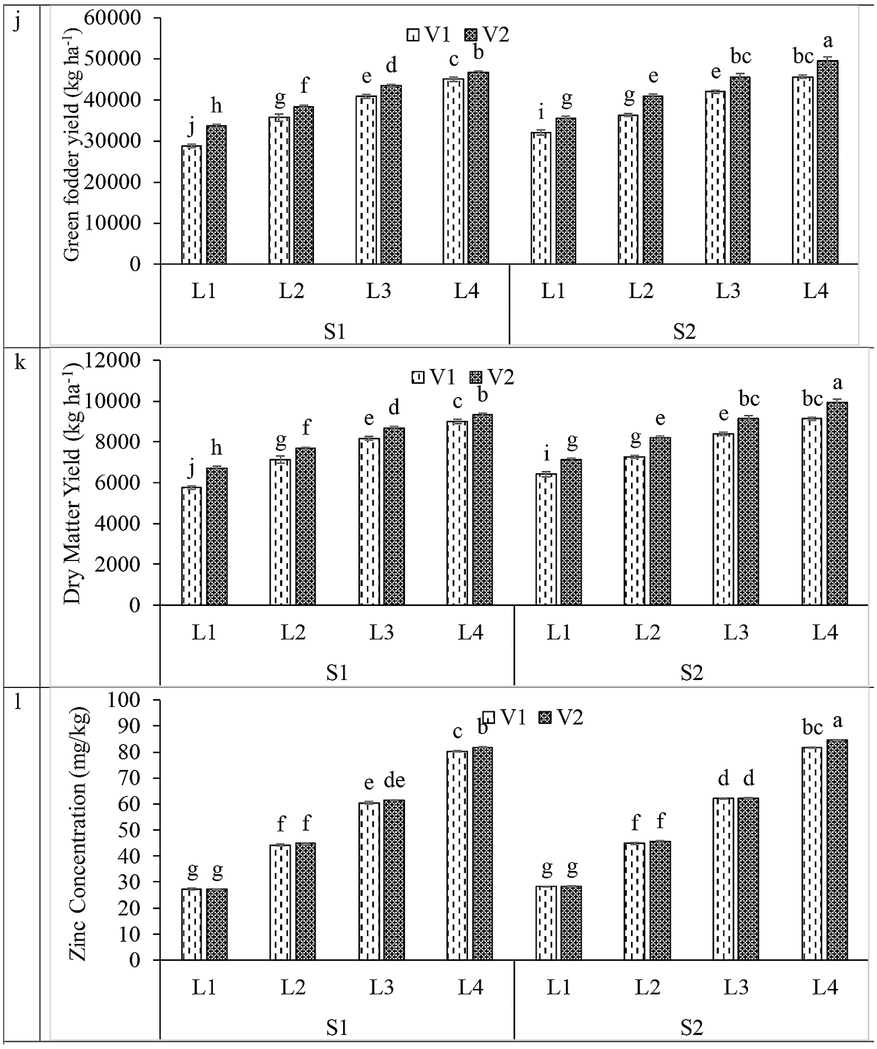

Figure 5. Effect of conventional and nano ZnO on green fodder yield, dry matter yield and zinc content of alfalfa.

V_1 = Supersonic FD 9 (Australian Origin) and V2 = Sargodha Lucerne 2002 (Pakistan Origin), S_1 = $ZnSO_4$ and S_2 = Zinc oxide nanoparticles, with four different zinc doses: L1 = 0 kg/ha, L2 = 3 kg/ha, L3 = 6 kg/ha, L4 = 9 kg/ha. Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$

dry matter yields of 5760, 7133.33, 8173.33, and 9000 at L1, L2, L3, and L4, respectively, whereas V2 recorded higher values of 6722.67, 7680, 8685.33, and 9333.33 at the corresponding zinc levels. Under S2, dry matter yield further increased, with V1 recording 6413.33, 7266.67, 8392, and 9128, while V2 produced 7120, 8186.67, 9120, and 9925.33 at L1, L2, L3, and L4, respectively. Across all zinc levels, S2 consistently resulted in greater dry matter yield than S1, and V2 outperformed V1 under both zinc sources. The highest yield of dry matter (9925.33) was obtained from the treatment combination V2 × S2 × L4, followed by V2 × S1 × L4 (9333.33) and V1 × S2 × L4 (9128), whereas the minimum yield (5760) was recorded in V1 × S1 × L1.

Total zinc content

Zinc content

As reflected in (Figure 5l) zinc content increased markedly with successive increases in zinc application, regardless of varieties and zinc sources. Under zinc source S1, zinc content in variety V1 increased from 27.33 at L1 to 44.27 at L2, followed by 60.47 at L3 and 80.33 at L4. Variety V2 followed the same increasing pattern, recording zinc contents of 27.13, 44.73, 61.40, and 81.80 at L1, L2, L3, and L4, respectively. A comparable trend was observed under zinc source S2, where variety V1 recorded zinc contents of 28.20, 44.80, 62.07, and 81.67 across L1 to L4, while variety V2 exhibited values of 28.33, 45.53, 62.20, and 84.53 at the corresponding zinc levels. Across both zinc sources, variety V2 generally accumulated slightly higher zinc content than variety V1, particularly at the higher application levels. Likewise, zinc source S2 consistently promoted greater zinc accumulation than S1, with the differences becoming more evident at L3 and L4. The highest zinc content (84.53) was obtained from the treatment combination V2 × S2 × L4, whereas the lowest value (27.13) was observed in V2 × S1 × L1. These results show that increasing zinc application directly improved the plants' zinc enrichment, with variety V2 showing the strongest capacity to accumulate zinc when given by source S2 at the highest zinc level.

Pearson correlation analysis

Relationships between shoot fresh weight (SFW), root fresh weight (RFW), number of leaves (NoL), chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), total chlorophyll (TChl), crude protein (CP), crude fiber (CF), crude fat (CFAT), green fodder yield (GFY), dry matter yield

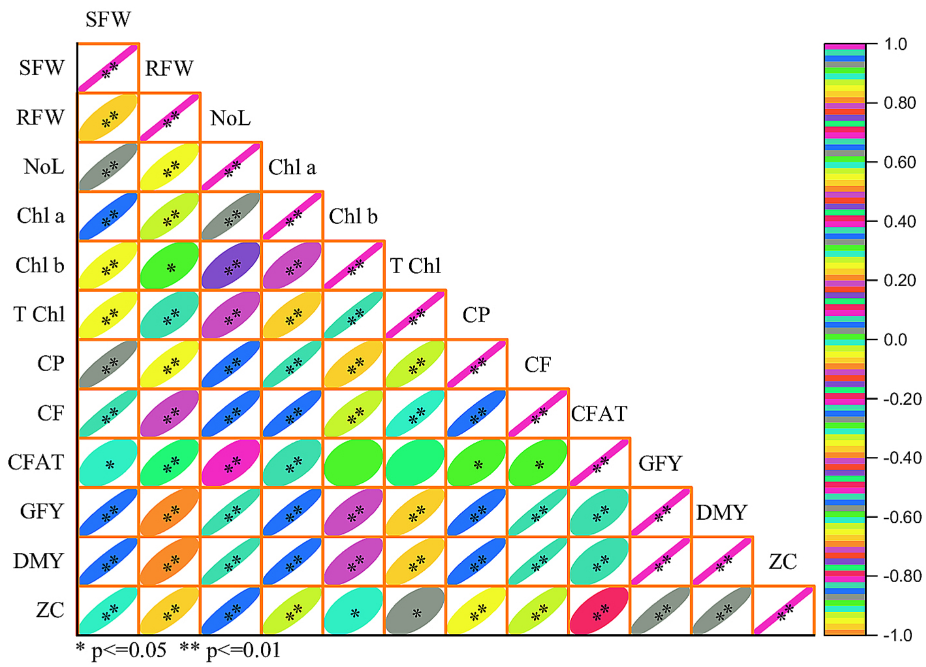

Figure 6. Pearson correlation matrix among growth parameters, biochemical attributes, quality attributes, yield attributes, total zinc content

(DMY), and zinc content (ZC) are shown in the correlation matrix (Figure 6). The positive correlations are denoted using colors in the blue/green range while the negative correlations are denoted using colors from the orange/red range. The thickness and form of the ellipse show the degree of correlation where narrow ellipses mean strong correlations. In general, the results demonstrated that most of the growth and quality attributes were positively correlated with each other. The traits such as chlorophylls (Chl a, Chl b, and TChl) had high positive correlations because of their physiological closeness. Green fodder yield (GFY) and dry matter yield (DMY) had positive correlations with various biomass attributes such as shoot fresh weight (SFW), root fresh weight (RFW), and number of leaves (NoL), indicating a positive association between forage production and vegetative growth. Zinc content (ZC) showed positive correlations with chlorophyll, crude protein, crude fiber, and yield traits, indicating that higher Zn concentration was associated with improved photosynthetic, quality, and yield attributes. On the other hand, very few trait pairs showed low or negative correlation, showing that there is little antagonism among these parameters. However, these correlations indicate associations among traits and should not be interpreted as evidence of causal relationships.

DISCUSSION

Growth attributes

Increase in shoot fresh weight with zinc application may be attributed to improved nutrient uptake, cell division, cell elongation, and vegetative growth. Better zinc availability increased nutrient absorption and assimilation, which resulted in an increase in fresh weight (Ijaz et al., 2023). Variety differences were due to genetic variations in nutrient use efficiency and growth capacity, giving the better variety the ability to yield more shoot biomass (Gaddam et al., 2026). The fresh weight of the roots was enhanced due to higher availability of zinc because of enhanced root development and nutrient acquisition (Goncalves et al., 2025). The better plant would have had a more efficient absorption of nutrients along with efficient root development, leading to an increase in the biomass of fresh roots. These responses may also have been supported by the complementary roles of N, P, and K, which are essential for balanced nutrient uptake, biomass production, and overall plant development. There was a rise in the number of leaves because of better availability of zinc, owing to efficient cell division, chlorophyll production, photosynthesis, and vegetative growth by IAA (Ulas et al., 2026).

Biochemical attributes

Improved zinc availability increased chlorophyll a content, likely due to enhanced chlorophyll biosynthesis and protection of photosynthetic pigments against oxidative damage (Ahmed et al., 2022). By lowering oxidative damage, improved zinc nutrition also contributes to the stability of chlorophyll. The superior species probably had a stronger ability for pigment production and nutrient usage, which led to a higher concentration of chlorophyll a. Chlorophyll b content also increased with zinc availability, possibly reflecting improved pigment stability and photosynthetic capacity. Additionally, zinc enhances pigment persistence by shielding photosynthetic pigments from oxidative deterioration (Dang et al., 2024; Khaliq et al., 2023).

Quality attributes

Because zinc is essential for nitrogen metabolism, enzyme activation, amino acid synthesis, and protein creation, crude protein content rose as zinc availability improved (Costa et al., 2023). While genetic variations in nutrient usage efficiency allowed the superior variety to accumulate larger crude protein content, enhanced zinc nutrition increased nitrogen assimilation and protein production (Sajjad et al., 2024). This response may have been further supported by adequate N availability, which is essential for protein synthesis. Due to improved cell wall development and the production of structural carbohydrates like cellulose and hemicellulose during plant growth, crude fiber content rose with increasing zinc availability (Zhao et al., 2025). Additionally, zinc promotes increased deposition of structural elements in plant tissues by supporting enzyme activity and the metabolism of carbohydrates. Zinc's function in fatty acid production, carbon metabolism, and enzyme activation affected the buildup of crude fat (Sajjad et al., 2025).

Yield attributes

Green fodder yield increased with improved zinc availability, primarily through greater assimilate accumulation, nutrient acquisition, and biomass production (Ulas et al., 2026). Zinc also promoted efficient assimilate production and translocation, leading to greater forage accumulation. The superior variety likely possessed higher nutrient use efficiency and biomass-producing potential, resulting in greater green fodder yield. Dry matter yield increased with improved zinc availability, reflecting greater carbohydrate accumulation and structural biomass formation (Suganya et al., 2020).

Total zinc content

Because of better zinc absorption, transport, and accumulation within plant tissues, zinc content rose as zinc availability increased (Stanton et al., 2022). Higher tissue zinc content was the outcome of better internal transport and food uptake efficiency brought about by an adequate supply of zinc. The greater zinc accumulation observed with nano-ZnO compared with conventional $ZnSO_4$ may be attributed to its smaller particle size, greater surface area, and enhanced solubility and availability in the soil–plant system. These properties can facilitate more efficient Zn release, root uptake, and translocation within the plant, resulting in greater Zn accumulation in alfalfa tissues. Thus, nano-ZnO may provide Zn more efficiently than conventional $ZnSO_4$ under the conditions of the present study. Despite the beneficial effects of nano-ZnO on zinc accumulation and alfalfa productivity, its repeated or long-term application may pose environmental concerns, including nanoparticle accumulation in soil and potential effects on soil microorganisms and nutrient cycling. Therefore, the field application of nano-ZnO should be based on appropriate application rates and monitored over the long term. Further multi-season studies are needed to evaluate its persistence, environmental fate, and potential ecological risks before recommending repeated large-scale application.

Pearson correlation analysis

The majority of the examined features had positive correlations, indicating that similar physiological and metabolic processes controlled their expression (Ferioun et al., 2023). Increased nutrient availability has been shown to improve nutrient assimilation and dry matter partitioning, all of which simultaneously improve growth, quality, and yield traits (Simkin et al., 2019). These coordinated reactions suggest that total plant performance is enhanced by favorable nutritional conditions rather than by specific features acting alone.

CONCLUSION

The results of the study showed that zinc biofortification greatly enhanced alfalfa's growth, physiology, quality, yield, and zinc accumulation, with nano zinc oxide consistently exceeding the traditional zinc supply across the majority of assessed parameters. The cultivar Sargodha Lucerne 2002 outperformed Supersonic FD 9 in terms of chlorophyll content, crude protein, biomass output, and forage zinc concentration when zinc application was increased. As a result, applying 9 kg ha^{-1} of nano ZnO to the soil can be suggested as a successful way to increase alfalfa productivity, forage quality, and zinc biofortification, which would improve cattle nutrition and promote sustainable forage production.

REFERENCES

1. Ahmed, N., Hussain, H. Z., Ali, M. A., Rahi, A. A., Saleem, M., Ahmad, F. (2022). Effect of zinc on chlorophyll contents, gas exchange attributes and zinc concentration in rice. *Pakistan Journal of Botany*, *54*(1), 17–24.
2. Amin, H., Habib, A., Asif, M., Hameed, M. U., Wassi, M., Manzoor, M., Sajjad, M. (2023). Effect of foliar application of boron, zinc and manganese on growth, yield and oil contents of sunflower (*Helianthus annuus* L.). *Asian Journal of Soil Science and Plant Nutrition*, *9*(4), 86–94.
3. Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant physiology*, *24*(1), 1.
4. Campisi, S. C., Khan, A., Zasowski, C., Bhutta, Z. A. (2021). Malnutrition. In *Textbook of Pediatric Gastroenterology, Hepatology and Nutrition: A Comprehensive Guide to Practice* (pp. 609–623). Cham: Springer International Publishing.
5. Costa, M. I., Sarmento-Ribeiro, A. B., Gonçalves, A. C. (2023). Zinc: from biological functions to

therapeutic potential. *International Journal of Molecular Sciences*, *24*(5), 4822.

6. Dang, K., Mu, J., Tian, H., Gao, D., Zhou, H., Guo, L., Shao, X., Geng, Y., Zhang, Q. (2024). Zinc regulation of chlorophyll fluorescence and carbohydrate metabolism in saline-sodic stressed rice seedlings. *BMC Plant Biology*, *24*(1), 464.
7. Elango, D., Kanatti, A., Wang, W., Devi, A. R., Ramachandran, M., Jabeen, A. (2021). Analytical methods for iron and zinc quantification in plant samples. *Communications in Soil Science and Plant Analysis*, *52*(10), 1069–1075.
8. Feier-David, S. R., Popescu, D., Bora, M., Paven, M. I., Peț, I. (2023). Research on the behavior of two autochthonous varieties of alfalfa under the conditions of tormac plain. *Scientific Papers Animal Science and Biotechnologies*, *56*(1), 72–72.
9. Ferioun, M., Srhiouar, N., Bouhraoua, S., El Ghachtouli, N., Louahlia, S. (2023). Physiological and biochemical changes in Moroccan barley (*Hordeum vulgare* L.) cultivars submitted to drought stress. *Heliyon*, *9*(2).
10. Gaddam, T., Pal, K., Rani, K., Saharan, R. P., Chaudhari, M. H. (2026). Evaluating genetic variation and potential for selection in growth and yield characteristics of wheat (*Triticum aestivum* L.) genotypes. *Discover Plants*, *3*(1), 74.
11. Goncalves, B., Santos, M., Silva, V., Rodrigues, A., Oliveira, I., Lopes, T., Sujeeth, N., Guinan, K. J. (2025). Biostimulants in fruit crop production: Impacts on growth, yield, and fruit quality. *Horticulturae*, *11*(12), 1452.
12. Goyal, K., Singh, N., Jindal, S., Kaur, R., Goyal, A., Awasthi, R. (2022). Kjeldahl method. *Advanced techniques of analytical chemistry*, *1*(1), 105.
13. Hadidi, M., Palacios, J. C. O., McClements, D. J., Mahfouzi, M., Moreno, A. (2023). Alfalfa as a sustainable source of plant-based food proteins. *Trends in Food Science & Technology*, *135*, 202–214.
14. Ijaz, B., Nazeer, S., Sajjad, M., Nadeem, M., Anwar, H., Idrees, M., Nawaz, M., Ramzan, H.N., Ihsan, F., Sherazi, S.M.W., Iqbal, U. (2023). Effect of various foliar applied micronutrients (Zn+ Fe+ Cu+ B) on growth and yield of wheat under Faisalabad condition. *Journal of Global Innovations in Agricultural Sciences*, *11*(2), 184–189.
15. Khaliq, A., Zafar, M., Sajjad, M., Hassan, M.U., Mudassar, M.A., Shakoor, M.A., Yasin, M., Niaz, S. (2023). Nitrogen use efficiency in sunflower (*Helianthus annuus* l.) influenced by various fertigation and bed planting techniques. *Pakistan Journal of Biotechnology*, *20*(2), 385–392.
16. Kiani, B.H., Arshad, I., Nazir, S., Saleh, I.A., Kiani, S.H., Zomot, N., Al-Qahtani, W.H., Alfuraydi, A.A., Abdel-Maksoud, M.A. (2024). Innovative approaches for sustainable zinc nutrition and crop yield enhancement in pea plants using zinc oxide nanoparticles. *Journal of Soil Science and Plant Nutrition*, *24*(3), 5829–5840.
17. Kiran, A., Wakeel, A., Mahmood, K., Mubaraka, R., Hafsa, Haefele, S. M. (2022). Biofortification of staple crops to alleviate human malnutrition: contributions and potential in developing countries. *Agronomy*, *12*(2), 452.
18. Naseer, M., Nawaz, A., Iram, A., Wishal, A., Waheed, K., Sajid, M., Gull, U., Sajjad, M., Rashid, A., Hassan, M.M. (2026). Effects of kitchen waste compost on soil CO_2-C emissions, microbial properties, enzyme activity and aggregate fractions: *Research Consortium Archive*, *4*(2), 1390–1414. https://doi.org/10.5281/zenodo.20094577
19. Ranjan, R., Chauhan, V. S., Chaudhary, M., Singh, K. K., Ahmed, S., Dwivedi, K. K. (2021). Expression of ZmZIP1 a gene involved in zinc transport after nutrient application in Oat (*Avena Sativa* L.). *Adv Biores*, *12*(2), 23–29.
20. Rashid, A., Sajjad, M., Arshad, M. U. (2023). Turmeric (*Curcuma longa* L.) in functional foods: Medicinal properties, nanoparticle applications and enhanced bioavailability: A comprehensive review. *Agriculture, Ecosystem and Economics*, *2*(1), 1–13.
21. Sajjad, M., Hussain, K., Wajid, S. A., Saqib, Z. A. (2024). The impact of split nitrogen fertilizer applications on the productivity and nitrogen use efficiency of rice. *Nitrogen*, *6*(1), 1.
22. Sajjad, M., Ijaz, B., Hur, M. (2026). 12 climate smart soil quality management strategies for resilient agriculture. *Sustainable Plant Nutrition and Soil Quality Management*, 199.
23. Sajjad, M., Ijaz, B., Khil, A., Ibtahaj, I., Hur, M., Azizi, N., Chughatta, A.R., Sajid, M., Rashid, A., Babar, S. (2025). Influence of different organic mulches on growth, yield and oil content of maize (*Zea mays* L.) hybrids and soil physical properties. *Journal of Ecological Engineering*, *26*(11).
24. Shakeel, A., Anjum, L., Ibni Zamir, S., Rizwan, M., Arshad, M., Mehmood, Q., Sajjad, M., Waqas, A. (2021). Irrigation water quality effects on CO_2 emissions along with crop and soil responses. *Pakistan Journal of Agricultural Sciences*, *58*(2), 637–642.
25. Simkin, A. J., López-Calcagno, P. E., Raines, C. A. (2019). Feeding the world: improving photosynthetic efficiency for sustainable crop production. *Journal of Experimental Botany*, *70*(4), 1119–1140.
26. Stanton, C., Sanders, D., Krämer, U., Podar, D. (2022). Zinc in plants: Integrating homeostasis and biofortification. *Molecular Plant*, *15*(1), 65–85.

27. Steel, R. G. D., Torrie, J. H. (1981). *Principles and procedures of statistics, a biometrical approach* (No. Ed. 2, p. 633pp).
28. Suganya, A., Saravanan, A., Manivannan, N. (2020). Role of zinc nutrition for increasing zinc availability, uptake, yield, and quality of maize (*Zea mays* L.) grains: An overview. *Commun. Soil Sci. Plant Anal*, *51*(15), 2001–2021.
29. Ulas, F., Ulas, A., Razzaghi, S., Yücel, Y. C. (2026). A comprehensive review on vegetable grafting: a sustainable approach to boost crop growth, development, and yield performance at high and low N status. *Journal of Crop Health*, *78*(2), 57.
30. Windsor, N., Boatwright, L., Boyles, R., Bridges, W., Rubiales, D., Thavarajah, D. (2024). Characterizing dry pea (*Pisum sativum* L.) for improved nutritional traits and the potential for biofortification. *Legume Science*, *6*(3), e250.
31. Zhao, Y., Shao, Y., Fan, S., Bai, J., Zhu, L., Zhu, Y., Xiao, X. (2025). Advanced modification strategies of plant-sourced dietary fibers and their applications in functional foods. *Foods*, *14*(15), 2710.