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Boron and pigment content in alfalfa affected by nano fertilization under calcareous conditions

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ABSTRACT

Boron is among the necessary nutrients for plant growth and yield production, and it can improve the nutritional properties of forage crops. However, at higher levels it maybe toxic adversely affecting plant growth and forage quality. Plant boron concentration is affected by different parameters including boron fertilization, soil, climate, plant species, etc. A new era in the field of plant fertilization is the use of nano technology. Accordingly, a factorial field experiment on the basis of a complete block design in six replicates was conducted in a 300-hactare agro-industry area. The objectives were to investigate the effects of different boron treatments on alfalfa (*Medicago sativa* L.): 1) boron concentration, and 2) pigment contents including chlorophyll *a*, b, total, and carotenoids. Six regions with different properties were selected for the experiment; the soil samples were collected using GPS. The experimental treatments including: 1) soil type (S1-S6), 2) boron sources including boric acid (B1) and nano boron fertilization (B2), and 3) number of spraying (zero-, one-, two-, and three-time) were tested. The nano structure of boric acid-copper in an aqueous medium was produced using the chemical reduction method. The results indicated that soil type, boron source and number of spraying significantly affected ($P \leq 0.01$) alfalfa boron concentration and pigment content. The mean of alfalfa boron concentration by B1 and B2 was 103.52 and 111.52% higher than control. The three-time spraying significantly increased B concentration related to the other spraying treatments ($P \leq 0.05$) as it resulted in a 207.81% increase compared with the control treatment. The three-time spraying resulted in the highest increase of pigment contents ($P \leq 0.05$) including chlorophyll *a*, b, total, and carotenoids compared with the other treatments. Although the highest boron and pigment contents were resulted by the three-time spraying, the combined use of B2 and the two-time spraying was the most optimum treatment (non-toxic) significantly increasing alfalfa boron and pigment contents ($P \leq 0.05$). It would be possible to produce alfalfa at large amounts and with suitable forage quality using boron nano fertilization (synthesized and tested in this research work) under calcareous conditions.

1. Introduction

Boron (B) is among the essential micronutrients for plant growth and yield production [1]. The most important sources of B for plant use are soil colloids including organic and mineral ones [2,3]. The critical range of B deficiency and B toxicity is little [4]. Accordingly, managing this nutrient and investigating its critical level in the soil and plant is not easy, and is of significance [5]. The critical level of B in the soil and plant has been investigated by different research work and the suitable range of 30–80 $mg\,kg^{-1}$ soil has been suggested for alfalfa [6].

Boron is mobile in some plants and immobile in some other plants [7]. It is because the mobility of B from the cellular outer space to the internal space is controlled by the formation of polypol complex, which is not available at required amount in some plants. Accordingly, providing B at sufficient levels in plants lacking polypol for improving pollination [8,9], fruit quality and storage [10] and plant tolerance under stress is a challenge [11]. It is hence essential to use a more efficient method of B fertilization resulting in higher B uptake and utilization by plant.

A new era in the field of plant fertilization and environmental remediation is the use of nano technology [12], which is really efficient and of economical significance compared with the ordinary methods. Research work has indicated that the use of nutrients at the nano scale (nano fertilization) can increase nutrient efficiency and results in the less use of fertilizer, increased yield production and decreased pollution of the environment [13–18].

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The effects of nano-fertilization with different elements have been investigated by researchers. Some research work has indicated the toxic, and some indicted the positives effects of nano-particles on plant growth. For example, Sharma and Uttam [19] analysed different morphological and biochemical properties of wheat (*Triticum aestivum* L.) seedlings subjected to copper (Cu) nano-fertilization using laser induced fluorescence and spectroscopy. The authors found the toxic effects of Cu on wheat seedlings by the increased level of lipid peroxidation. In another similar study, Sharma et al. [20] indicated the positive effects of gold nano-particles on the growth of wheat seedlings. The results indicated the enhancement of proteins, lipids, cellulose, hemicelluloses, pectin, and lignin in the leaves of wheat seedlings affecting the rigidity and functionality of cellular wall. Bharti et al. [21] found both the negative and positive effects of titanium dioxide nanoparticles on the growth of garlic (*Allium sativum*) seedlings. According to the results such nano-particles decreased plant root length, and increased plant aerial growth, chlorophyll *a*, chlorophyll b, total chlorophyll, carotenoid, and quercetin in the leaves. Sharma and Uttam [22] investigated the toxic effects of Al2O3 nano-particles on the growth and physiology of wheat seedlings and indicated the mechanisms by which the seedlings can resist the stress. The Al2O3 nano-particles resulted in the increased production of proteins, lipids, hemicelluloses, cellulose, pectin and lignin in wheat seedlings as the mechanisms, which may enhance plant resistance under AL2O3 stress.

The passage of nano particle into the cellular membrane is a function of cellular diameter, and the smaller nano particles can easily cross the membrane [23,24]. The movement of nano particle into the plant cell is controlled by the carrier proteins, membrane complexes, and root exudates [25,26]. The uptake of nano particles by plant cuticle and stomata and their subsequent transport to the other cells by plasmodesmata has also been shown by research work [27–29].

The nano particle in the cytoplasm moves to the cytoplasmic organelles and it is subsequently utilized in the cellular metabolism [24]. The coated boric acid on the metal nano particle (size of 10–1000 nano meter) compared with the fertilizer at the macro scale, can easily cross the cuticle and move all over the plant plasma membrane. This action is done in the absence of polysaccharide complexes indicating that the metal nano particle catalyzes the crossing of compounds into the cellular plasma membrane [7].

Alfalfa is among the important forage crops and its growth and yield production is significantly affected by B. The favorable effects of B on crop growth and yield production has been indicated by different research work, however to our knowledge there is not any data on the use of B nano fertilization affecting alfalfa physiology under calcareous conditions. Accordingly, the objective of the research work was to investigate the effects of B resources (boric acid and nano fertilization) and number of spraying on alfalfa B concentration and pigment content in different calcareous soils.

2. Materials and methods

2.1. Synthesis of nano boric acid

The nano structure of boric acid-copper in an aqueous medium was produced using the chemical reduction method [7,30]. Accordingly, 0.496 g $CuCO_4$ was added to one liter of water (distilled twice), heated to the temperature of 80 °C, and was mixed for one minute. Boric acid at 0.12 g was poured into the solution and mixed for two minutes. Sodium borohydride at 1.26 g was dropped into the solution, which was heated until the color changed. The solution was allowed to cool down to the room temperature and was mixed for 50 min. To avoid any changes in the nano structure, the production and consumption (spraying) of nano boric acid was done simultaneously [7,30]. The concentration of B in the nano fertilizer was used as the reference for the production of B from boric acid. To make the experimental treatments comparable and avoid any error in the production of nano fertilizer, Cu (at the amount used for the production of nano fertilizer) was also mixed with the boric acid solution.

2.2. Research area

The study region is an 800-ha agro-industry center located at Sharif-Abad, Ghazvin Province, Iran. It is located at the 36° 10′ and 47″ Northern latitude and 50° 9′ and 52″ Eastern longitude. The products of the region are wheat, barley, alfalfa and forage corn. The research work was conducted on alfalfa in a 300-ha area.

2.3. Soil sampling and analyses

For the collection of soil samples in the field, a network sampling method was used including 40 soil samples using 100 × 100 squares to the 30 cm depth, and the sampling positions were recorded using GPS. The collected soil samples were analysed in the lab and after being air dried and passed through a 2-mm sieve, their physical and chemical properties determined [31]. The amount of extractable B was measured using the hot water method [32,33]. The soil texture [34], soil pH [35], equivalent calcium carbonate [36] and soil organic matter [37] were also determined.

2.4. Experimental design

Accordingly, six regions with different properties were selected for the experiment, and after selecting the experimental points, the experiment was conducted as a three way factorial on the basis of a completely randomized block design in six replicates. The experimental treatments including: 1) six different soils (S1-S6), 2) two different B sources including boric acid (B1), nano B fertilizer (B2) and control (B3), and 3) four different number of spraying (zero-, one-, two- and three-time) during the growth period were tested.

2.5. Use of treatments

The first spraying was done right after the first harvest and before the growth of the second harvest (Apr. 28th, 2016) followed by the second and the third spraying, two and four weeks after the first spraying, respectively. The plants were finally harvested following the complete plant growth (June, 5th, 2016). The extraction of plant B was done by the dry digestion method [38] and the method of azomethine-H., using spectrophotometer at the 430 nm wavelength [39]. The amounts of chlorophyll *a*, b and total were also determined [40].

2.6. Statistical analyses

Data were subjected to analysis of variance using SAS, and the single and the interaction effects of experimental treatments on alfalfa B concentration and pigment content were determined. Using Duncan's multiple range test the differences between the means were recognized at $P \leq 0.05$.

3. Results

3.1. B concentration

The TEM picture of nano structure of boric acid-copper is presented in Fig. 1. The Zeta analysis of the particle distribution indicated that although the particles with the diameter of 5–10 nm were present, the dominant size of the particles were in the range of 10–1000 nm, and the highest accumulation was related to the range of 100 nm (Fig. 2). Table 1 presents the physical and chemical properties of the selected soils with a wide range of properties. For example, the soil B is in the range of 0.79–1.67 mg kg^{-1}, clay: 18–34%, and organic matter: 1.38–3.10 %.

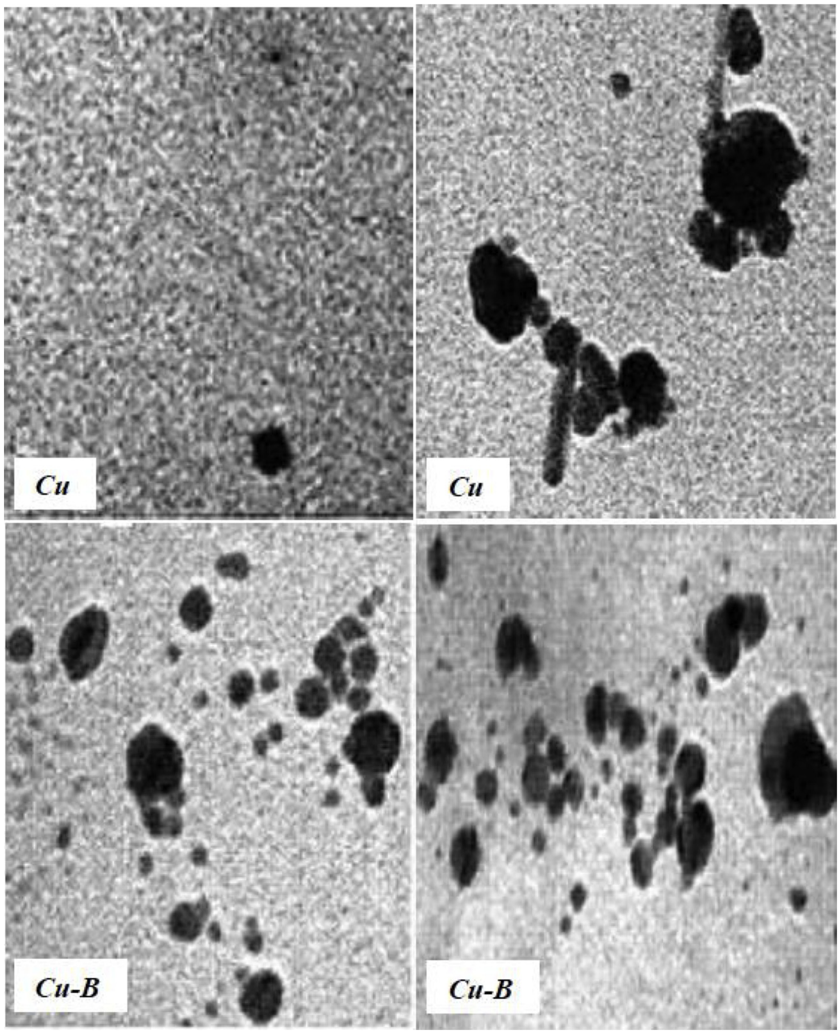

Fig. 1. The TEM pictures of synthesized nano Cu and nano Cu-boric acid (Cu-B).

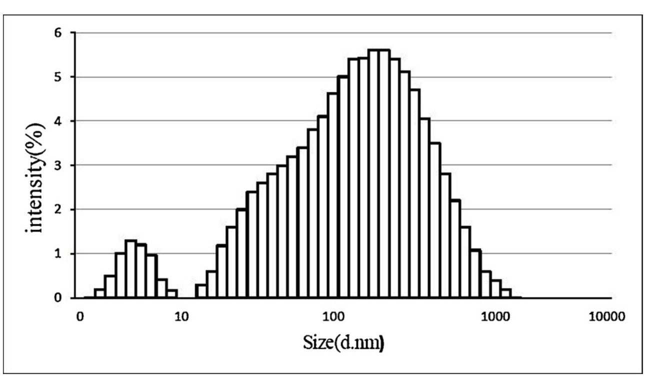

Fig. 2. The Zeta analysis indicating the distribution of nano particles according to their diameter.

Analysis of variance indicated that the effects of soil type, B source, number of spraying, and their interactions significantly ($P \le 0.01$) affected alfalfa B concentration (Table 2). The mean differences also indicated that soil type significantly affected the alfalfa concentration as the highest and the least B concentrations were resulted by S3 and S6, respectively. Accordingly, the B concentration in S3 was 1.6 times higher than that of S6. Similarly, the B sources also significantly affected alfalfa B concentration and the highest and the least values were resulted by B2 and B3, respectively. The alfalfa B concentration increased by 111.52 and 103.52% in B2 and B1, compared with control. However, the significant difference in plant B concentration resulted by B2 and B1 was only 3.93% (Fig. 3A).

The interaction effects of soil and B source indicated that the highest B concentration was resulted by the combination of S3 and B2 and the least concentration by S1 and control treatment. The use of B2 resulted in the significant increase of alfalfa B concentration in S1, S2, S3, S4, S5 and S6 by 344.9, 55.97, 110.64, 97.90, 69.64 and 41.63% compared with the control treatment. The corresponding values for B1 were equal to 346.26, 46.89, 74.44, 136.54, 42 and 42.7% (Fig. 3A).

The three-time spraying significantly increased B concentration related to the other spraying treatments ($P \le 0.05$) as a 207.81% increase was resulted by the three-time spraying compared with the control treatment. However, the difference in B concentration between the one-time spraying and the two-time spraying was only 9.38% significantly different from the three-time spraying. The interaction effects of soil and number of spraying indicated that the combination of S1 and the three-time spraying resulted in the highest (638.27% increase related to the control, $P \le 0.05$) and S1 and control resulted in the least B concentration (Fig. 3B). The highest and the least B concentration were resulted by the combination of the three-time spraying and B2, and by the zero-time spraying and B1, respectively. The corresponding increases by B2 related to the control treatment using one-, two- and three-time spraying were equal to 50.66, 76.48 and 207.44% and by B1 50, 52.40 and 208.18% (Fig. 3C).

The interactive effects of soil, B source, and number of spraying

Table 1
The physical and chemical properties of the selected soils.

Soil	Texture	pH	Clay %	Silt	Sand	Organic matter	$CaCo_3$	Extractable B mg. kg^{-1}
S1	C.L.	8.52	26	20	54	2.13	3.44	1.29
S2	L.	8.70	25	31	44	2.20	4.29	0.79
S3	L.	7.57	26	37	37	3.10	5.46	1.46
S4	S.L.	8.07	18	15	67	1.38	5.35	0.87
S5	C.L.	8.49	34	38	28	2.34	5.88	1.67
S6	L.	8.16	26	42	32	1.62	6.73	1.02
Mean		8.24	25.40	28.61	41.72	2.05	5.07	1.18

C.L.: Clay loam, L.: Loam, S.L.: Sandy loam.

Table 2
Analysis of variance indicating the effects of different experimental treatments on alfalfa B uptake.

S.V.	d.f.	Mean
Soil	5	26535.89**
BS	1	325.86**
NS	3	71900.82**
Soil x BS	5	876.27**
Soil x NS	15	5898.09**
BS x NS	3	329.14**
Soil x BS x NS	15	435.29**
CV (%)		2.73

S.V.: source of variation, d.f.: degree of freedom, BS: B source, NS: number of spraying, CV: coefficient of variation.
** : significant at P ≤ 0.01.

indicated that the highest B concentration in alfalfa was resulted by S1 and the combination of the three-time spraying and B2, and the least by S1, S6 and the zero-time spraying and B3. The use of the two-time and three-time spraying in S1 resulted in a 233.17 and 660.59% increase in B concentration compared with control. In the S2 soil there was not a significant difference between one-time and two-time spraying on B concentration, however the three-time spraying significantly increased B concentration using B2 (140.57% increase compared with control) related to B1 (112.51% increase related to the control treatment) (P ≤ 0.05). Using the S3 soil the highest B concentration was resulted by B2 as one-, two- and three-time spraying increased B concentration by 69.96 (54.89% by B1), 104.17 (33.24% by B2), and 157.79% (135.19% by B2), respectively. The highest and the least B concentration in S5 was related to B2 and control treatments at 71.07 and 34.46 mg kg^{-1}, respectively. Accordingly, the use of one-, two- and three-time spraying, significantly increased B concentration in B1 by 9.4, 19.69 and 96.94%, and in B2 by 44.60, and 58.09, 106.22%, compared with control (P ≤ 0.05). The results indicated that there is not any significant difference on B concentration between S4 and S6 using B1 and B2 (Fig. 3D).

3.2. Pigments

The analysis of variance indicated that different experimental treatments significantly affected alfalfa pigment contents including chlorophyll *a*, chlorophyll b, total chlorophyll *a*nd carotenoids (P ≤ 0.01) (Table 3).

The pigment contents in different soil types were significantly different as S1 resulted in higher chlorophyll *a*, chlorophyll b, total chlorophyll *a*nd carotenoids of 130.35, 209.09, 173.96, 101, and 54% than S4. According to the results, plants grown in S1 had the highest B concentration and pigment content. Different B sources significantly affected alfalfa pigment contents as B2 and B3 resulted in the highest and the least pigment content, respectively. chlorophyll *a*, b, total and carotenoids by B2 were higher at 50.16, 85.96, 7.33 and 41.62% and by B1 at 30.42, 56.39, 45.05 and 21.34%, compared with control (Fig. 4).

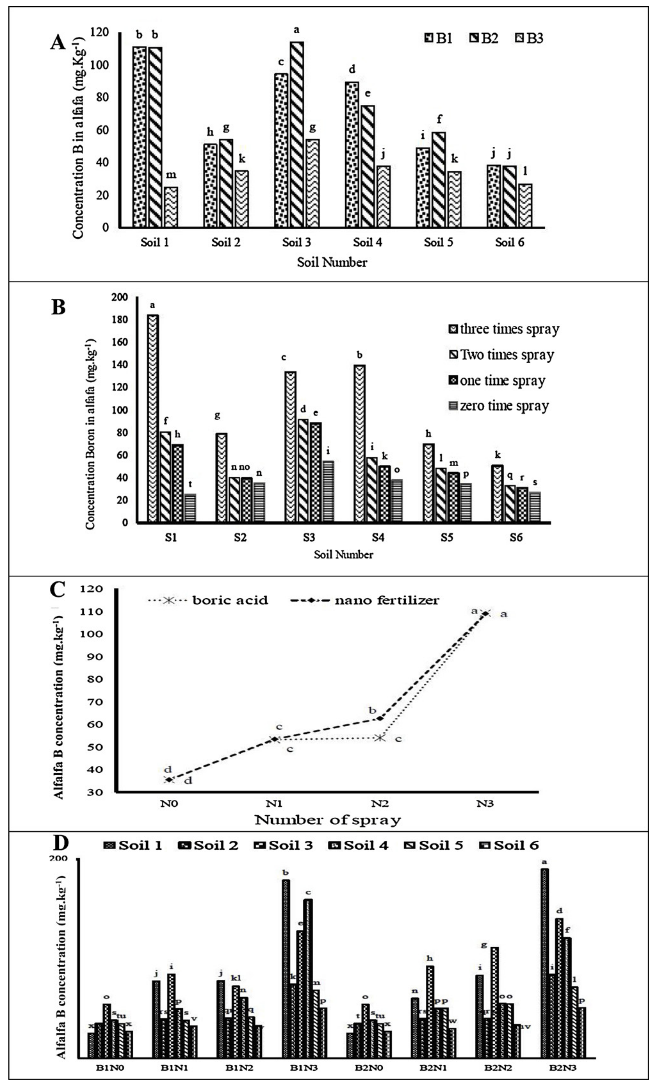

Fig. 3. The interactive effects of **(A)** soil and B source, **(B)** soil and number of spraying (N0-N4), **(C)** B source and number of spraying, and **(D)** soil, B source and number of spraying on alfalfa B concentration (B1, B2 and B3 stand for boric acid, nano B fertilizer and control). Columns followed by different letters are significantly different using Duncan's multi range test at P ≤ 0.05.

The interaction effects of soil type and B source revealed that the increase of chlorophyll *a*, b, total and carotenoids in S1 compared with control using B1 was equal to 19.64, 52, 38.64, 11.58% and using B2 was equal to 29.27, 56.14, 45.05 and 25.11%. The corresponding increases for S4 using B1 was 21.90, 19.45, 20.37 and 8.3% and using B2 was 44.76, 113.57, 79.62 and 20.52%. The results indicated that both B1 and B2 treatments resulted in the highest chlorophyll and

Table 3
The effects of experimental treatments on alfalfa pigment concentration.

S.V.	d.f.	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll	Carotenoids
Soil	2	0.017^{**}	0.067^{**}	0.151^{**}	256.294^{**}
BS	2	0.001^{**}	0.005^{**}	0.009^{**}	16.714^{**}
NS	2	0.003^{**}	0.016^{**}	0.034^{**}	48.291^{**}
Soil x BS	4	$2.79 \times 10^{-4**}$	0.001^{**}	0.002^{**}	6.678^{**}
Soil x NS	4	0.005^{**}	0.014^{**}	0.035^{**}	85.701^{**}
BS x NS	4	$2.79 \times 10^{-4**}$	0.002^{**}	0.004^{**}	4.783^{**}

** :Significant at $P \leq 0.01$.

carotenoids contents (Fig. 4).

The three-time and zero-time spraying resulted in the highest and the least plant pigments as three-time spraying resulted in higher chlorophyll *a*, b, total and carotenoids at 63.10, 110.77, 89.97 and 51.93% compared with control. The corresponding values for the two-time spraying were equal to 29.12, 52.13, 41.8 and 26.15% and for the one-time spraying 28.48, 50.37, 41.10 and 16.37%. According to the interaction effect of soil type and number of spraying, the highest pigments were resulted by the three-time spraying and S1 (the highest B concentration) with the increase of 92.53, 143.05, 121.95, 84.80% compared with the control treatment (Fig. 5). The interaction effect of B source and number of spraying revealed that the three-time spraying and B1 increased chlorophyll *a*, b, total and carotenoids by 56.13, 117.94, 89.69 and 46.25% and the three-time spraying and B2 by 69.03, 111.28, 90.25 and 57.93% compared with control (Fig. 6).

4. Discussion

Boron (B), as an important micronutrient, determines plant growth and yield production by affecting different physiological processes including the pigment contents. Plant chlorophyll content is a major parameter influencing the important process of photosynthesis. Accordingly, providing plants with the adequate amount of B is a must for the optimum production of crop plants under different conditions including calcareous soils. The ordinary methods of B fertilization are not efficient, environmentally and economically, and may not supply adequate amounts of B for plant use.

The important physiology and functioning of boron in plant include: 1) the mobility of boron in plant is affected by polyol transport molecules, 2) the polysaccharides complex of cell wall (pectin polymers) determines the boron crosstaking, 3) boron affects membrane processes, 4) membrane functioning is rapidly altered by boron, 4) the metabolic pathways are influenced by boron in plant by affecting the apoplastic proteins and the manganese related enzymes, 5) boron alleviates the toxic effects of aluminum on root growth of dicotyledonous plants, 6) boron regulates pollen germination, 7) boron enhances fruit yield and quality, 8) plant stress genes are induced by boron deficiency and toxicity [1,11].

Arif et al. [41] found that the combined use of B and Zn significantly increased rice growth and yield as well as chlorophyll content and B absorption and assimilation. The deficiency of B can directly and indirectly affect the photosynthesis process [42]. The rate of photosynthesis in different soybean genotypes increased by using boron and molybdenum compared with control [43].

The results of this research work indicated that soil type can

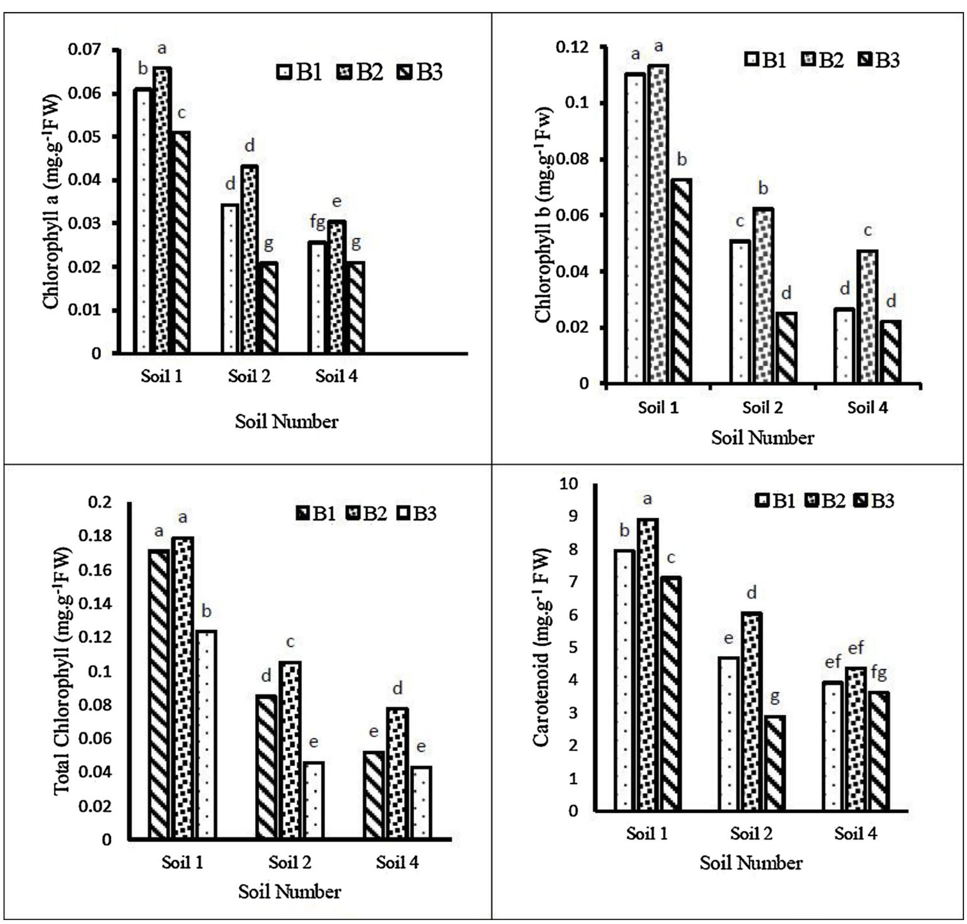

Fig. 4. The interactive effects of soil and B source on alfalfa pigment content. B1, B2 and B3 stand for boric acid, nano fertilizer and control, respectively. Columns followed by different letters are significantly different using Duncan's multi range test at $P \leq 0.05$.

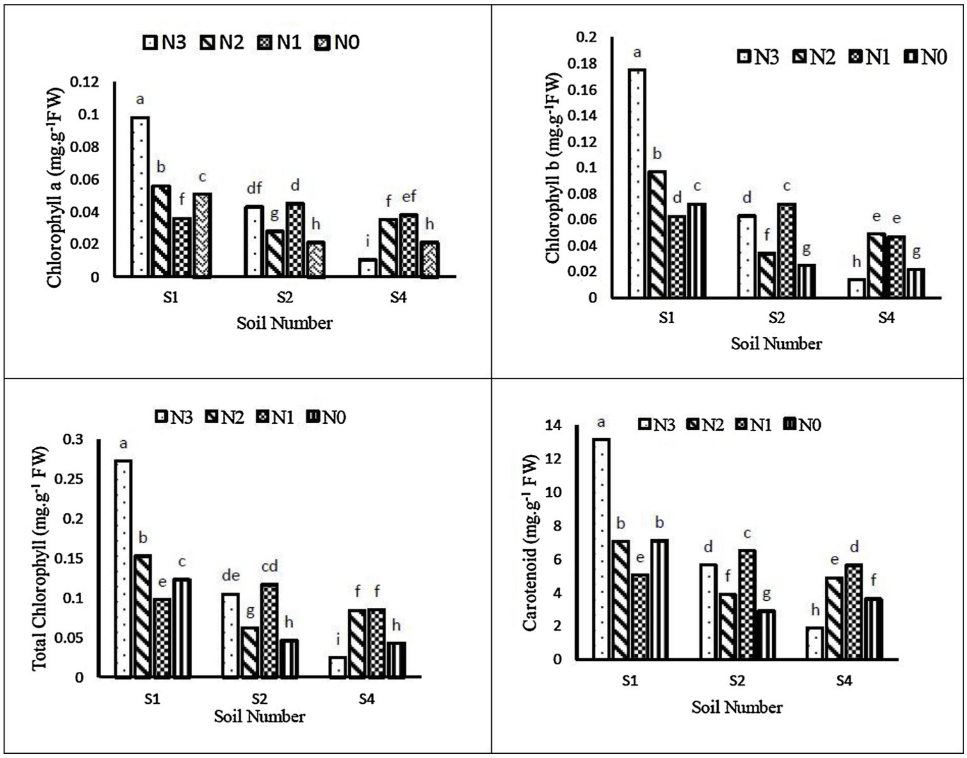

Fig. 5. The interactive effects of soil and number of spraying (N0-N4) on alfalfa pigment content. Columns followed by different letters are significantly different using Duncan's multi range test at P ≤ 0.05.

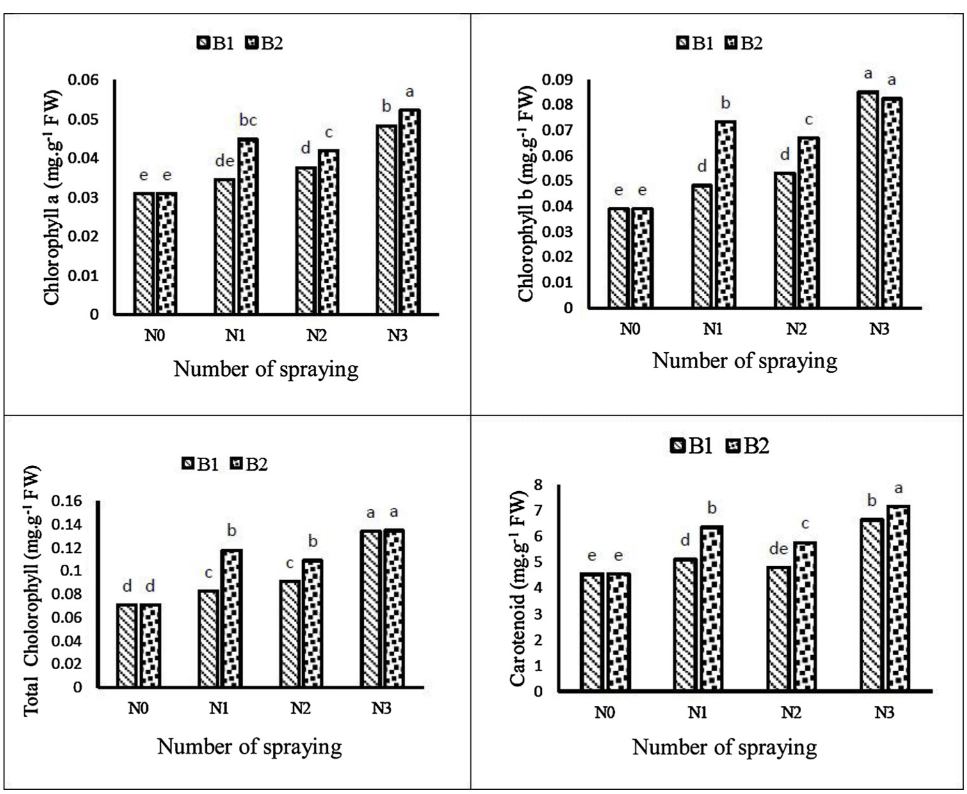

Fig. 6. The interactive effects of B source and number of spraying on alfalfa pigment content, B1 and B2 stand for boric acid and nano fertilizer, respectively. Columns followed by different letters are significantly different using Duncan's multi range test at P ≤ 0.05.

significantly affect alfalfa B concentration and pigment contents. The differences in soil B concentration are the result of pH, soluble B concentration, soil texture and moisture, soil minerals, the type and amount of organic matter, the type of exchangeable ions, plant species, etc. (Table 1). The surface adsorption of B increases with increasing pH from 3 to 9. Aluminum and iron hydroxide, calcium carbonate, organic matter and clay minerals are the main sources of B adsorption [44–48].

The efficient uptake of B by plant is an important factor determining B utilization by plant. Although boric acid can provide plant with its required amounts of B, it can adversely affect N fixation process in legumes including alfalfa, as it may not be as efficient as B nano fertilization. The research work by Deb [7] indicated that the boric acid on the metal nano particles (10–1000 nm), is able to easily move across the cellular membrane in the absence of the polysaccharide complexes, and as a result the metal nano particles act as a catalyst for the movement of nutrients across the cellular membrane. According to Liu et al. [49] the coating and cementing of nano and nano composite are able to regulate the diffusion of nutrients from the fertilizer particles. Different research work has indicated that the use of nano particles for providing plants with their adequate amounts of nutrients is an efficient method environmentally and economically [15].

The number of spraying also significantly affected alfalfa B concentration and pigment contents. The three-time spraying resulted in the highest alfalfa B concentrations (110 mg kg^{-1}), much higher than plant requirement, which in some cases were at toxic levels, indicating that the three-time spraying is not a suitable number of spraying. However, this was not the case for the two-time spraying as it resulted in the most optimum and efficient level of B for alfalfa use. The reason that there was not a significant difference between the one-time spraying and the two-time spraying on B concentration can be due to the significant plant growth till the two-time spraying and the subsequent dilution effect. However, during the three-time spraying the plant did not have a significant growth and hence the concentration of B significantly increased.

The sufficient level of B in alfalfa is equal to 30–80 mg kg^{-1}, indicating that the plants in the control treatment (35.42 mg kg^{-1}) were B deficient. The increases of 50.66 and 50% in B concentrations by B2 and B1, using the one-time spraying revealed that there was not any significant difference between the two treatments. This can be due to the fact that during the initial stages of plant growth (little plant biomass) plant is not demanding to B. However, the B2 (62.5 mg kg^{-1}) treatment significantly increased B concentration in plant over the B1 treatment (54 mg kg^{-1}) ($P \leq 0.05$).

It should also be mentioned that there was not a significant difference between B concentration at the one-time and the two-time spraying. Research work has shown the higher efficiency of nano fertilizer at less amount, compared with chemical fertilizer [50–52]. The formulation of nano-structure can behave more precisely, and gradually release the nutrients for plant use in response to environmental conditions and biological demands [51]. According to the results, different soil types were B deficient under control treatment, and the two-time spraying treatment increased the alfalfa B concentration to a favorable level by B2 (62.54 mg kg^{-1}) and B1 (54 mg kg^{-1}). Will et al. [53] found that depending on B concentration in plant the absorption and mobility of B in plant is affected.

5. Conclusion

This research work was conducted in a large agro-industry research area (300 ha) investigating the effects of different soil types (from six different regions), B sources and number of spraying on alfalfa B concentration and pigment content including chlorophyll *a*, b, total and carotenoids. According to the results the most optimum combination of B and number of spraying was B2 (B nano fertilization) and the two time spraying significantly enhancing alfalfa B concentration and pigment content. Such results are of environmental and economical significance for the optimum production of alfalfa. It would be possible to produce alfalfa at large amounts and with suitable forage quality using B nano fertilization (synthesized and tested in this research work) under calcareous conditions.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Main finding

It would be possible to produce alfalfa at large amounts and with suitable forage quality using boron nano fertilization (synthesized and tested in this research work) under calcareous conditions.

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