Colegio de Postgraduados

Interaction between nitrogen doses and alfalfa (*Medicago sativa* L.) incorporation in lettuce (*Lactuca sativa* L.) production

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ABSTRACT

Objective: To determine the optimal nitrogen dose combined with alfalfa in the growth, yield, and ion concentration in the sap of the lettuce leaf.

Design/Methodology/Approach: We used a completely randomized experimental design, with a 2×5 factorial arrangement and nine repetitions in each treatment. The treatments consisted of five nitrogen doses (200, 250, 300, 350, and 400 kg ha^{-1}) and two soil conditions (with and without alfalfa).

Results: Aerial fresh weight (AFW), aerial dry weight (ADW), end-to-end diameter of the shoot (EDS), maximum diameter of the shoot (MDS), shoot weight (SW), and yield were higher when a 200-300 kg ha^{-1} dose of N was applied along with alfalfa. This application had a similar effect to the 300 kg dose of N ha^{-1}, applied without the addition of alfalfa. The concentration of nitrates $\left(NO_3^-\right)$ in the sap decreased with the addition of alfalfa and 200 kg of N ha^{-1}. The concentration of potassium (K^+) was higher with or without the addition of alfalfa and 250 kg of N ha^{-1}. The concentration of calcium (Ca^{2+}) was higher in the plants that received a 400-kg dose of N ha^{-1}, whether alfalfa was included or not.

Study Limitations/Implications: The lack of equipment prevented the determination of the nitrogen available in the soil.

Findings/Conclusions: The incorporation of alfalfa into the soil is a good alternative to improve lettuce production and reduce the use of nitrogen fertilizers.

Keywords: Growth, quality, yield, nitrate reduction.

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INTRODUCTION

Nitrogen (N) is an essential macronutrient for the growth and development of plants. It is part of amino acids, nucleic acids, chlorophyll, amino enzymes, and alkaloids. In addition, it is involved in the cell multiplication and differentiation processes (Balta *et al.*, 2015; Rodríguez *et al.*, 2020). However, the excessive use of nitrogen fertilizers is directly related to increased production costs and environmental pollution problems; therefore, it is essential to have information that helps to determine the adequate amounts needed to obtain maximum crop yields (Rodríguez *et al.*, 2020). Nitrogen fertilization in the

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Mexican agricultural sector is related to a major source of greenhouse gas emissions. The said fertilization emits nitrous oxide and contributes 12.3% of the total greenhouse gas emissions (Gonzáles and Camacho, 2017).

One of the alternatives to reduce the use of nitrogen fertilizers is the rotation of nitrogen-fixing species (Fabaceae) with the horticultural crop of interest. Alfalfa (*Medicago sativa* L.) (Fabaceae) can fix atmospheric nitrogen (N_2) through a symbiotic association with bacteria of the *Rhizobium* genus, converting this element to a form that the plants can assimilate (Delgado, 2015; Guzmán and Montero, 2021). It plays an important role in agriculture, as a consequence of its contribution to the reduction of the use of fossil energy and the emission of greenhouse gases produced by the use of nitrogen fertilizers (Ángeles and Cruz, 2015). Biological nitrogen fixation makes an important contribution to the N in the soil (Celaya and Castellanos, 2011). The incorporation of Fabaceae residues into the soil can increase its N content (Magaña *et al.*, 2020). In addition, the use of this type of plants as green manure in horticultural crops supplies the N that the plant demands, favoring the reduction of the excessive use of fertilizers, improving fertility, and reducing soil erosion (Castro *et al.*, 2018; Magaña *et al.*, 2020). This process contributes to the mitigation of environmental impact. Some studies report that the N provided by alfalfa is usually enough to achieve the optimal yield of some Poaceae (grasses) in the first cycle; however, sometimes the demand of the plant can also be satisfied through minimum amounts of nitrogen fertilizer. In addition to the N provided by alfalfa, previous studies have shown that soil structure can also be improved (Yost *et al.*, 2014). The process of biological nitrogen fixation in alfalfa through symbiosis could increase from 24 to more than 584 kg of N ha^{-1} year^{-1} and, in some cases, supply up to 90% of the plant's requirements (Angeles and Cruz, 2015).

For its part, lettuce is one of the most consumed vegetables worldwide, given its low level of calories, sodium, and fat. Likewise, it is an excellent source of fiber, iron, folic acid, and vitamin C (Kim *et al.*, 2016). Therefore, our objective was to determine which nitrogen dose (combined with the incorporation of alfalfa) is optimal to improve growth, yield, and the ion concentration in the sap of the lettuce leaf.

MATERIALS AND METHODS

The study was carried out from August 2019 to September 2021 in the Campo Experimental of the Departamento de Horticultura de la Universidad Autónoma Agraria Antonio Narro, in Saltillo, Coahuila, Mexico (25° 21' 22.5" N, 101° 02' 08.7" W), located at an altitude of 1,610 m. The study site has a loamy soil, with an apparent density of 1.03 g cm^{-3} and a pH of 7.94. The average annual temperature is 16.8 °C and the climate is dry and semi-arid. In August 2019, alfalfa seeds cv. Giant were sown, in previously prepared 80-cm wide beds. The sowing density was 30 kg ha^{-1} of seed, and a broadcast sowing method was used. A drip irrigation system was installed, using a Toro® Aqua-Traxx™ tape of 6,000 calibers, with 20 cm between the emitters and a hydraulic flow rate of 1.05 L h^{-1}. The plants were irrigated 3 h per day, twice a week, and no fertilization was applied during the cycle of this crop. The last three alfalfa cuts were incorporated by hand into the beds where the lettuce seedlings were subsequently transplanted and covered with black polyethylene plastic mulch (1.20 m wide, 90 μm caliber, TACSA®).

Vegetal material

Romaine lettuce seedlings var. Capitata were used. This variety forms a tight shoot through the inner leaves and has wide and sinuous leaves. Its growth cycle lasts for approximately 60 d. The transplant was carried out on July 20, 2021, with seedlings that had five leaves. The distance between plants was 30 cm and the rows were established 80 cm apart from each other. Consequently, the planting density was adjusted to 50,000 plants ha^{-1}.

Treatments

The treatments consisted of five N doses (200, 250, 300, 350, and 400 kg ha^{-1}) and two soil conditions (with and without alfalfa). Their combination resulted in a total of 10 treatments. A 150 kg ha^{-1} dose of phosphorus (P) and a 250 kg ha^{-1} dose of potassium (K) were applied to each treatment. The source of those nutrients was: ammonium sulfate (20.5-00-00-24), monoammonium phosphate (12-61-0), and potassium nitrate (12-00-45). The fertilization dose was divided according to the phenological stage of the crop. The fertilization for each treatment began seven days after the transplant and it was carried out twice a week. The irrigation frequency depended on the water requirements of the plants, but on average it was carried out for 2 h every two days. The experimental design used was completely randomized with a 2×5 factorial arrangement and each treatment consisted of nine repetitions.

Parameter determination

The variables were measured on September 30, 2021, at 70 days after transplant (dat). To determine the aerial fresh weight (AFW), the aerial part was separated from the plant and weighed with a Rhino® 0.01 precision electronic scale. The outer leaves were removed, leaving only the edible part, to determine shoot weight (SW), using the said scale. The end-to-end diameter of the shoot (EDS) and maximum diameter of the shoot (MDS) were determined with an Insize® 1205-3002S vernier calliper. Subsequently, the aerial part of the plant was put in brown paper bags and then placed in a Blue M® POM-246F™ drying oven at a temperature of 65 °C for 72 h. Finally, the aerial dry weight (ADW) was recorded. To determine the ion concentration of nitrate $\left(NO_3^-\right)$, potassium (K^+), and calcium (Ca^{2+}), three plants were selected per repetition in each treatment. The samples were collected from 10:00 to 11:00 am and, once they were properly identified, they were stored in a cooler. The samples were a combination of the external and internal part of the lettuce. The ions from the petiole cell extract were determined with the Horiba® LAQUAtwin™. The yield (t ha^{-1}) was obtained multiplying the SW by the planting density (50,000 plants ha^{-1}).

Statistical analysis

The data obtained were subjected to an analysis of variance (ANOVA) and the mean comparison was carried out with Tukey's test (α of $p \leq 0.05$), which was carried out with the 2008 version of the InfoStatstatistical software.

RESULTS AND DISCUSSION

Aerial fresh weight (AFW), aerial dry weight (ADW), shoot weight (SW), end-to-end diameter shoot (EDS), and maximum diameter shoot (MDS) were significantly affected by the addition of the amendment. These same parameters were significantly affected by different N doses, as well as by the interaction of both factors, except for the EDS (Table 1). The plants developed in the soil to which alfalfa had been added showed higher parameters than those developed on the soil without this amendment (Table 1). The highest AFW, ADW, SW, and MDS were obtained with the 300-kg dose of N ha^{-1}. These variables showed a decrease with higher and lower doses, while the EDS was higher with 250 kg of N ha^{-1} than with the higher and lower doses applied (Table 1).

Both the concentrations of nitrate $\left(NO_3^-\right)$ and calcium (Ca^{2+}) ions in the lettuce sap, as well as the yield (ha^{-1}) were significantly affected by the addition of alfalfa. However, potassium (K^+) did not show a significant difference (Table 2). In these same parameters, significant differences were recorded with the application of different doses of N. Likewise, these variables were influenced by the interaction between the two factors (Table 2). The highest NO_3^- concentration was recorded in the plants which received an application of 400 kg of N ha^{-1}, while those to which doses of 200, 250, and 350 kg of N ha^{-1} were applied showed a decrease in the concentration of the said ion. The lowest NO_3^- concentration was obtained with the 300-kg dose of N ha^{-1}. The Ca^{2+} concentration was higher when 400 kg of N ha^{-1} were added, but it decreased when a 200-350 kg dose of N ha^{-1} was applied. Regarding K^+, higher concentrations were

Table 1. Effect of the amendment (alfalfa) and N addition in the soil on the aerial fresh weight (AFW/PFA), aerial dry weight (ADW/PSA), shoot weight (SW/PC), end-to-end diameter shoot (EDS/DPC), and maximum diameter shoot (MDS/DEC) of romaine lettuce var. Capitata.

Alfalfa	AFW (g plant^{-1})	ADW (g plant^{-1})	SW (g plant^{-1})	EDS (cm)	MDS (cm)
With alfalfa	1144.86a	38.66a	777.99a	13.87a	16.47a
Without alfalfa	752.93b	31.55b	430.21b	10.20b	12.32b
Anova p≤	0.0001	0.0001	0.0001	0.0001	0.0001
N (kg ha^{-1})					
200	854.67c	36.77b	520.46b	11.82abc	14.03b
250	1001.55b	34.86b	623.11ab	12.72a	14.64ab
300	1164.94a	41.56a	741.91a	12.65ab	15.40a
350	868.88c	34.31b	624.67ab	11.69bc	14.30ab
400	854.43c	28.05c	510.33b	11.29c	13.60b
Anova p≤	0.0001	0.0001	0.0001	0.0003	0.0058
Interaction p≤	0.0001	0.0003	0.0171	0.3283	0.0086
CV (%)	8.49	11.39	19.50	7.15	8.04

≤ 0.01 and 0.05 = Significant. ≥ 0.05 Not Significant. Anova = analysis of variance. Interaction = Alfalfa×N. CV = coefficient of variation. Means with different letters indicate significant effects, according to Tukey's multiple comparison test (p≤0.05). Aerial fresh weight (AFW), aerial dry weight (ADW), end-to-end diameter of the shoot (EDS), maximum diameter of the shoot (MDS), shoot weight (SW).

recorded with the 250-kg dose of N ha-1, while the concentration of the ion decreased with other doses (Table 2).

The yield was higher with 300 kg of N ha^{-1}; however, adding higher and lower doses resulted in a lower parameter (Table 2). Overall, most of the parameters evaluated in this research were higher in plants grown in soil with alfalfa than in soils grown without alfalfa.

The AFW was higher when 200 kg of N ha^{-1} were applied, and alfalfa was added to the soil. As the N dose increased, the AFW slightly decreased. The plants to which only chemical fertilization was applied recorded the maximum AFW with the 300-kg dose of N ha^{-1}; meawhile, higher and lower doses showed a decrease in the said variable (Figure 1). A similar behavior was observed in the ADW (Figure 2). Overall, plants that grew in soil to which alfalfa had been added showed higher values than those that were nurtured only with nitrogen fertilization. The increases in the AFW and ADW of the plants developed with alfalfa may be caused by the greater availability of N in the soil to which alfalfa had been added. Magaña *et al.* (2020) and other authors indicate that the N content in the soil can be increased through the incorporation of Fabaceae residues. This measure can diminish nitrogen fertilization in certain crops, such as corn (Cela *et al.*, 2011). In addition, N favors the growth and development of plants, given its structural function and its involvement in cell multiplication and differentiation (Rodríguez *et al.*, 2020). For their part, the diminishing of the AFW and ADW of the plants that developed only with nitrogen fertilization (200-250 kg doses of N ha^{-1}) are likely the result of the insufficient amount of N added for the achievement of an adequate growth, which decreases the productive potential (Rodríguez *et al.*, 2020). Martínez *et al.* (2008) reported considerable reductions

Table 2. Effect of the amendment (alfalfa) and N addition in the soil on the concentration of nitrate $\left(NO_3^-\right)$, calcium (Ca^{2+}), potassium (K^+) ions in the sap and yield of romaine lettuce var. Capitata.

Alfalfa	NO_3^- (mg kg^{-1})	Ca^{2+} (mg kg^{-1})	K^+ (mg kg^{-1})	Yield (t ha^{-1})
with alfalfa	3176.67b	156.60a	1230.00a	38.90a
without alfalfa	6280.00a	122.00b	1258.33a	21.51b
Anova p≤	0.001	0.001	0.180	0.001
N (kg ha^{-1})				
200	4800.00b	145.00b	1250.00b	26.02b
250	4550.00b	124.25c	1442.50a	31.16ab
300	3800.00c	122.50c	1158.33bc	37.10a
350	4700.00b	79.75d	1120.00c	31.23ab
400	5791.67a	225.00a	1250.00b	25.52b
Anova p≤	0.001	0.001	0.001	0.001
Interaction p≤	0.001	0.001	0.001	0.017
CV (%)	6.21	7.27	4.49	19.50

≤ 0.01 and 0.05 = Significant. ≥ 0.05 Not Significant. Anova = analysis of variance. Interaction = Alfalfa × N. CV = coefficient of variation. Means with different letters indicate significant effects according to Tukey's multiple comparison test ($p \leq 0.05$).

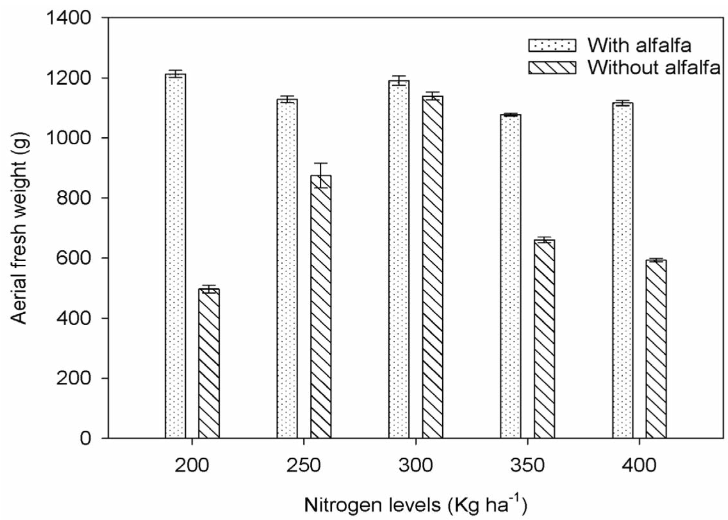

Figure 1. Effect of the interaction between alfalfa incorporation and different N doses on the aerial fresh weight (AFW) of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

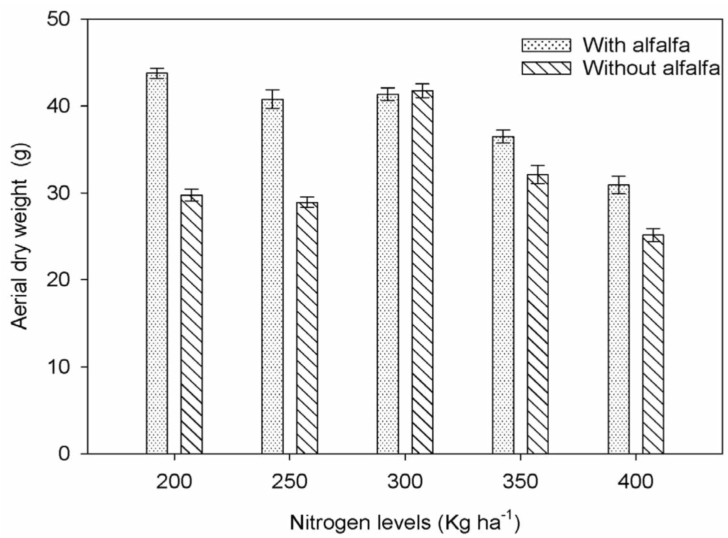

Figure 2. Effect of the interaction between alfalfa incorporation and different doses of N on the aerial dry weight (ADW) of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

in the fresh and dry weight of the cape gooseberry fruits nurtured with minimal amounts of N.

The EDS was higher (250 kg of N ha^{-1}) in plants developed on soil with alfalfa. However, as the N dose increased, the EDS tended to decrease slightly. In the case of plants developed only with nitrogen fertilization, a higher EDS was found (300 kg of N ha^{-1}). Likewise, the application of higher and lower doses resulted in a slight decrease in EDS (Figure 3). This behavior was similar in the MDS (Figure 4). The EDS and MDS reductions on plants that were nurtured only with nitrogen fertilization may be related to the amount of N applied. In this regard, Martínez *et al.* (2008) observed that applying

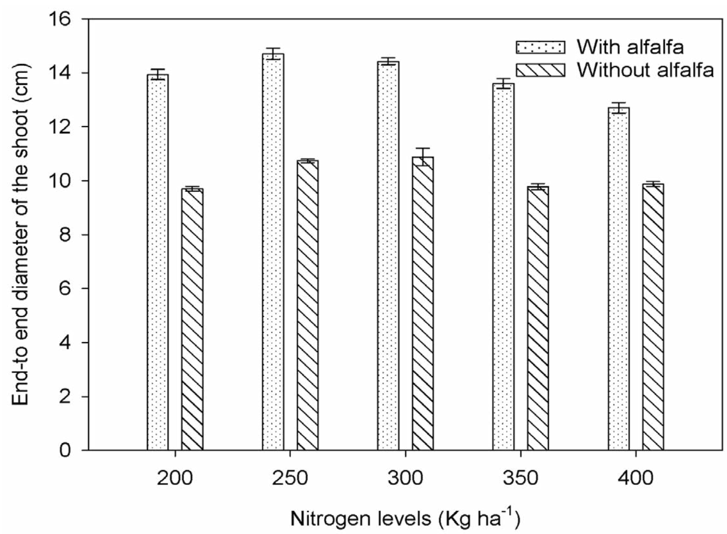

Figure 3. Effect of the interaction between alfalfa incorporation and different doses of N on the end-to end diameter of the shoot (EDS) of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

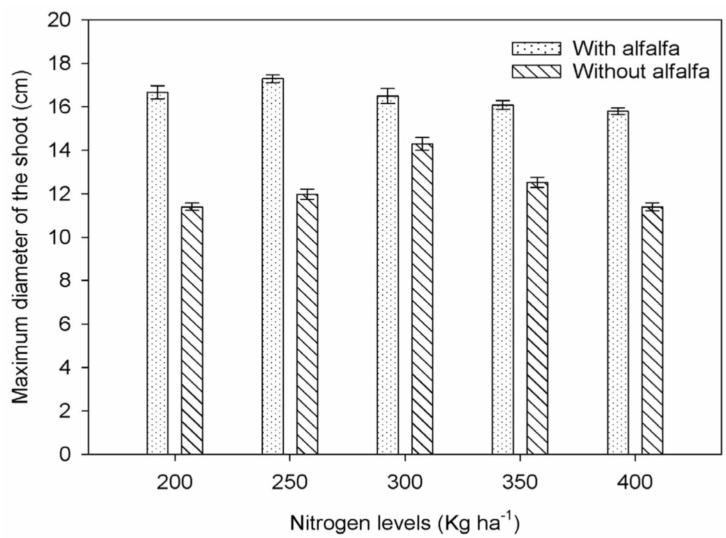

Figure 4. Effect of the interaction between alfalfa incorporation and different doses of N on the maximum diameter of the shoot (MDS) of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

minimum amounts of N during fertilization had a negative effect on the end-to-end and maximum diameter of cape gooseberries with respect to complete fertilization, indicating that they had an inadequate growth and low productivity. Likewise, the size of the lettuce showed a considerable increase when alfalfa was added.

The SW was higher in the plants that were developed in soil with alfalfa and with application of 200 and 300 kg of N ha^{-1}. However, when higher N doses were added, the SW tended to decrease. In the plants that were nurtured only with nitrogen fertilization, the highest SW was recorded with the 300-kg dose of N ha^{-1}, because the addition of lower and higher doses led to a decrease of this parameter (Figure 5). Overall, the plants that developed in the soil to which alfalfa and different doses of N were incorporated had higher SW than those that only received nitrogen fertilization. These results could possibly be attributed to the successful supplementation by alfalfa of the N demanded by the plant. In this regard, Samaddar *et al.* (2021) indicate that alfalfa increases the availability of N in the soil and, consequently, increases crop yield. Ángeles and Cruz (2015) point out that, in some cases, alfalfa can supply up to 90% of the N requirements of the plant. The results indicate that alfalfa can help to reduce the use of nitrogen fertilizers, since maximum yields can be achieved by applying minimum amounts of N.

The lowest NO_3^- concentration in the sap was obtained with the 200-kg dose of N ha^{-1} in the plants that were developed in the soil with alfalfa. As the N dose increased, the NO_3^- concentration also tended to increase. Overall, lower NO_3^- concentrations were found in plants that were grown in soil to which alfalfa had been added than in those soils were grown without alfalfa. The accumulation of NO_3^- in plants depends on the amount of N applied (Liu *et al.*, 2014) and the size of the biomass of the plants. Ollúa *et al.* (2016) pointed out that as the N dose increases, the concentration of NO_3^- in the loose-leaf lettuce also tends to increase. These findings match the results found in this research for the plants that were developed in the soil to which alfalfa had been added. Regarding the plants that were developed only with nitrogen fertilization, a higher NO_3^- concentration was recorded when a 200-400 kg dose of N ha^{-1} was applied. However, supplying 200, 250, 300, and 350 kg doses of N ha^{-1} tended to decrease the concentration of the said ion and the lowest concentration was obtained with 300 kg of N ha^{-1} (Figure 6). This behavior can be directly related to biomass, since the plants that received a lower amount of N had lower biomass. A similar effect occurs in plants nurtured with high amounts of N: the concentration of this ion in the sap tends to be higher. However, Ollúa *et al.* (2016) point out that applying >75 kg doses of N ha^{-1} to butterhead lettuce reduces the NO_3^-

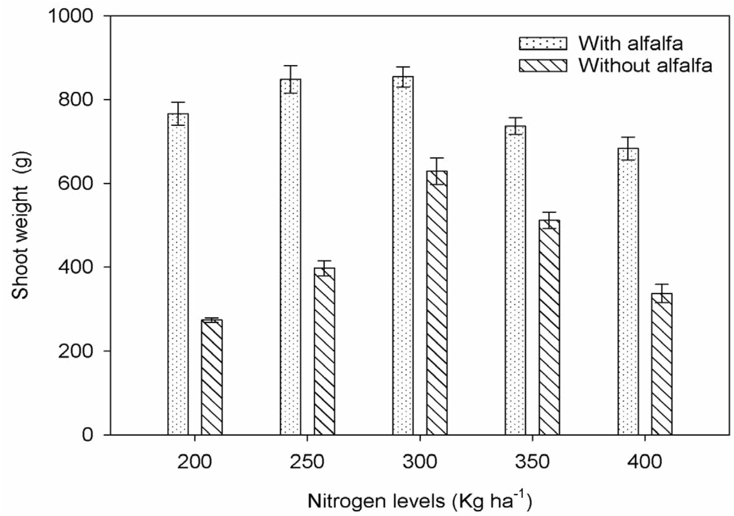

Figure 5. Effect of the interaction between alfalfa incorporation and different doses of N on the shoot weight (SW) of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

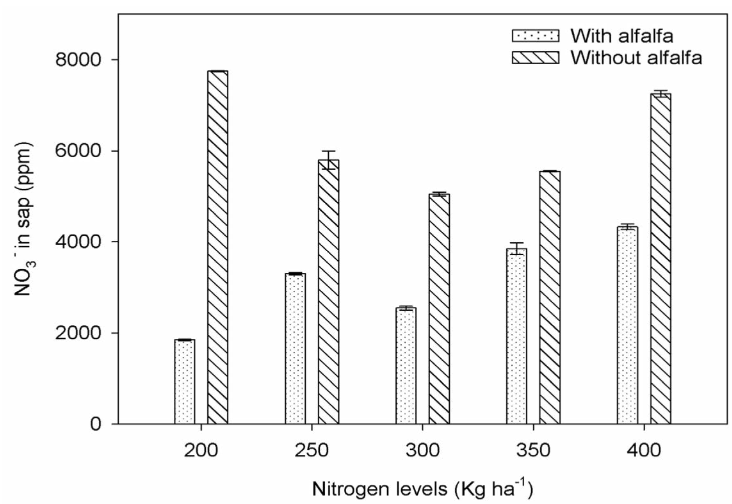

Figure 6. Effect of the interaction between alfalfa incorporation and different doses of N on the NO_3^- concentration of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

concentration in the sap. This behavior is possibly the result of a dilution effect, since the plants obtained a higher biomass.

The highest K^+ concentration was registered with the 250-kg dose of N ha^{-1}. This phenomenon was observed in the plants that developed on the ground both with and without alfalfa. Supplying higher and lower doses resulted in a slight reduction in the concentration of the said ion (Figure 7). In this regard, Gaona *et al.* (2020) report higher K^+ concentrations in sweet granadilla leaves with high doses of N, possibly as a consequence of the synergism that exists between them. However, the concentrations of this ion may have been affected by the concentration-dilution phenomenon: low and high N doses led to lower biomass, while the 300-kg dose of N ha^{-1} resulted in a higher biomass. Therefore, the concentration of said ion was negatively affected.

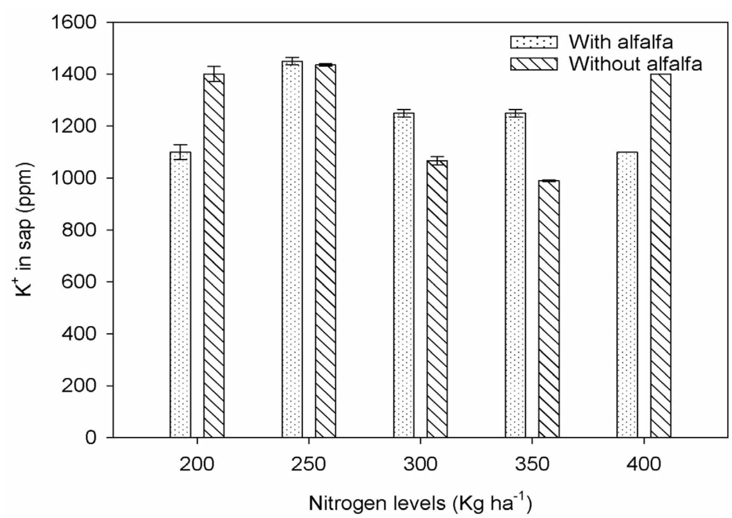

Figure 7. Effect of the interaction between alfalfa incorporation and different doses of N on the potassium (K^+) concentration of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

The Ca^{2+} concentration was higher when 400 kg of N ha^{-1} were added; this phenomenon was observed in the plants developed in soil with and without alfalfa. However, when 200 and 350 kg doses of N ha^{-1} were added, a decrease in the concentration of said ion was registered (Figure 8). Hernández and Rubilar (2012) reported reductions in the foliar concentration of Ca^{2+} in shoots of *Pinus radiata* hedges as the dose of N increased. The high Ca^{2+} concentrations resulting from the 400-kg dose of N ha^{-1} are possibly due to a concentration effect. The plants nurtured with the said dose had lower biomass, as a consequence of the high amounts of N applied.

The yield was higher with the 200- and 300-kg doses of N ha^{-1} combined with alfalfa. However, it tended to decrease when higher doses were added (Figure 9). The increases

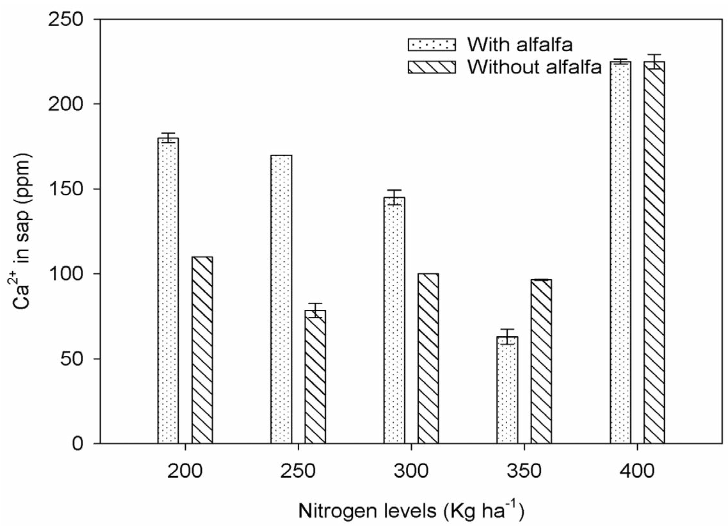

Figure 8. Effect of the interaction between alfalfa incorporation and different doses of N on the calcium (Ca^{2+}) concentration of romaine lettuce var. Capitata. The bars indicate the standard error of the means.

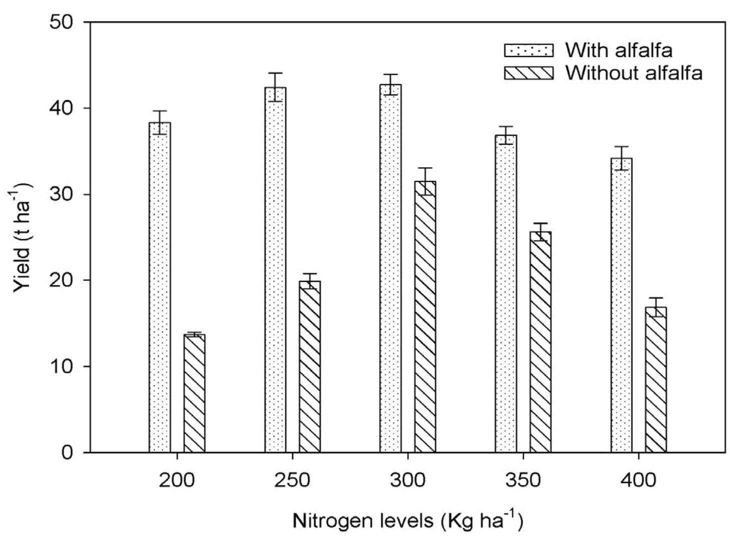

Figure 9. Effect of the interaction between alfalfa incorporation and different doses of N on the romaine lettuce var. Capitata yield. The bars indicate the standard error of the means.

in yield with the minimum doses of N may be caused by the presence of enough N for the plants in the soil, as a result of the incorporation of alfalfa and its N fixation. For their part, Cela *et al.* (2011) found high yields of corn grain with doses of 0 and 150 kg of N ha^{-1} after using alfalfa. Their results are similar to those found in this study, which suggests that the N provided by alfalfa can supply the subsequent crop's demand for this nutrient.

The decrease of this parameter in the plants that were developed without alfalfa and with the application of 200 and 400 kg of N ha^{-1} can be attributed to both the lack and excess of N. In this regard, García *et al.* (2021) did not find a significant difference in corn yield when 200 and 400 kg doses of N ha^{-1} were applied; therefore, they suggest that 400 kg of N ha^{-1} is an excessive dose and causes a low use of N by the plant. Therefore, applying large amounts of N does not necessarily increase crop yield (Sotomayor *et al.*, 2017; Rodríguez *et al.*, 2020). In this regard, Grijalva *et al.* (2016) observed a negative effect on wheat yield when a 460-kg dose of N ha^{-1} was applied. For their part, Sanchez *et al.* (2006) indicate that the impact of excessive N applications on biomass production is more negative than when this nutrient is deficient. A similar effect was observed in this research, especially in plants that were nurtured only with nitrogen fertilization: the maximum yield was recorded with 300 kg of N ha^{-1}. Meanwhile, Grijalva *et al.* (2016) recorded a maximum wheat yield with a 230-kg dose of N ha^{-1}. For their part, Rodríguez *et al.* (2020) found higher yields with a 200-g dose of N $plant^{-1}$ in passion fruit cultivation, while lower doses had a negative impact on the growth and development of the plants.

CONCLUSIONS

The use of alfalfa favors growth, yield, and ion concentration in lettuce plants. The addition of alfalfa and a 200-kg dose of N ha^{-1} decreases the NO_3^- concentration in the leaf sap. The Ca^{2+} concentration was higher in plants that were developed with alfalfa. The incorporation of alfalfa to the soil, combined with 200- and 300-kg doses of N ha^{-1} increased the growth and yield of the lettuce plants. These findings suggest that the incorporation of alfalfa to the soil is a good alternative to improve lettuce production and reduce the supply of nitrogen fertilizers.

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