

## Effect of different nitrogen fertilizer formulations on the morphological and phytochemical characteristics of various lettuce (*Lactuca sativa* L.) cultivars

### Uticaj različitih formulacija azotnih đubriva na morfološke i fitohemijske karakteristike različitih sorata zelene salate (*Lactuca sativa* L.)

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#### ABSTRACT

This study investigated the effects of different nitrogen mineral fertilizer formulations on the morphological and phytochemical traits of five butterhead lettuce (*Lactuca sativa* L.) cultivars grown under protected conditions. The experiment was conducted in a greenhouse during the winter-spring growing season. The analyzed cultivars included Genezis, Zeralda, Majska kraljica, Atrakcija, and Nansen. Three types of nitrogen fertilizers—ammonium sulfate, ammonium nitrate, and urea—were applied at a rate of 100 kg N/ha. The observed parameters included head weight, head height, vitamin C content, and nitrate concentration in the leaves. Significant differences were found among cultivars for all analyzed traits. The highest head weight was recorded in the cultivar Genezis, while the lowest was in Nansen. Head height ranged from 10.25 cm (Genezis) to 12.05 cm (Nansen). Vitamin C content varied between 18.88 mg/100 g (Genezis) and 23.55 mg/100 g (Nansen). The highest nitrate accumulation was observed in Majska kraljica, and the lowest in Nansen, with ammonium nitrate fertilization resulting in the highest average nitrate content. The interaction between cultivar and fertilizer type significantly influenced the measured traits, suggesting that each cultivar responds differently to nitrogen fertilization. These findings underscore the importance of selecting appropriate nitrogen fertilizers and cultivars to optimize yield, nutritional quality, and food safety in lettuce production.

**Keywords:** ammonium sulfate, ammonium nitrate, urea, head weight, nitrate content

#### APSTRAKT

U ovom istraživanju ispitivan je uticaj različitih mineralnih azotnih đubriva na morfološke i hemijske osobine pet različitih sorata zelene salate (*Lactuca sativa* L.), tip puterica gajenih u zaštićenom prostoru. Ogljed je izveden u plasteniku tokom zimsko-prolećne proizvodne sezone. Analizirane sorte bile su: Genezis, Zeralda, Majska kraljica, Atrakcija i Nansen. Primenjena su tri različite formulacije azotnih đubriva: amonijum sulfat, amonijum nitrat i urea, u dozi od 100 kg/ha azota. Praćeni su parametri masa glavice, visina glavice, sadržaj vitamina C i koncentracija nitrata u listovima. Utvrđene su značajne razlike između ispitivanih sorata za sve praćenje osobine. Najveću masu glavice imala je sorta Genezis, a najmanju sorta Nansen. Visina glavice kretala se od 10,25 cm (Genezis) do 12,05 cm (Nansen). Sadržaj vitamina C varirao je od 18,88 mg/100 g (Genezis) do 23,55 mg/100 g (Nansen). Najveća akumulacija nitrata zabeležena je kod sorte Majska kraljica, a najmanja kod Nansena, pri čemu je đubrenje amonijum nitratom rezultovalo najvećim vrednostima nitrata. Interakcija između sorte i tipa azotnog đubriva značajno je imala uticaja na analizirane osobine, ukazujući na postojanje specifične reakcije genotipova na đubrenje. Rezultati ukazuju na važnost pravilnog izbora azotnog đubriva radi optimizacije prinosa, kvaliteta i bezbednosti hrane.

**Ključne reči:** amonijum sulfat, amonijum nitrat, urea, masa glavice, sadržaj nitrata

## INTRODUCTION

Consumption of leafy green vegetables represents an important component of the human diet, as they are rich in phytochemical compounds—vitamins, minerals, antioxidants, and other protective factors essential for maintaining health. Lettuce (*Lactuca sativa* L.) is among the most popular leafy vegetables worldwide. Although it has a low energy value, lettuce is an excellent source of dietary fiber, vitamin C, iron, phenolic compounds, flavonoids, phenolic acids, and other secondary metabolites that positively influence human health (M'hamdi et al., 2014; Govedarica-Lučić and Perković, 2015; Khurshied et al., 2018; Bewuket and Shewaye, 2020; Geneva et al., 2021).

Numerous studies have highlighted the influence of various factors on the nutritional characteristics of lettuce, including ecological factors (e.g., fertilization, lighting) and biological ones such as cultivar and growth stage (Kim et al., 2016; Khan et al., 2018; Geneva et al., 2021; Bošković-Rakočević et al., 2023). In addition, clinical studies have indicated a correlation between regular lettuce consumption and a reduced risk of certain diseases, including colorectal cancer (Fernandez et al., 1997).

The phytochemical profile of lettuce leaves varies significantly depending on genotype as well as on the applied cultivation practices (Govedarica-Lučić and Perković, 2015; Kim et al., 2016; Geneva et al., 2021; Bošković-Rakočević et al., 2023). Despite its nutritional value, one of the main quality criteria for vegetables is low nitrate content (Ryszard et al., 2017). Leafy vegetables have a pronounced ability to accumulate nitrates (Catherine et al., 2017), with excessive use of nitrogen fertilizers being the main cause of this phenomenon.

Lettuce belongs to the group of leafy vegetables capable of accumulating very high levels of nitrates in their leaves (Yaseen and Hajos, 2021). The issue arises because a portion of the ingested nitrates is reduced to nitrites in the oral cavity by bacteria of the *Salvia* genus, and these nitrites may pose a serious health risk to humans (Harte et al., 1991; Hord et al., 2009). High levels

of nitrites in the body lead to the oxidation of iron in the hemoglobin molecule from the ferrous ( $\text{Fe}^{2+}$ ) to the ferric ( $\text{Fe}^{3+}$ ) form, resulting in the formation of methemoglobin, which is incapable of transporting oxygen in the blood (Blom-Zandstra, 2008). For this reason, the European Union has established regulations setting maximum limits for nitrate content in leafy vegetables to 2,500–4,500 mg/kg of fresh weight, depending on growing conditions and methods (Santiago et al., 2008; Liu et al., 2014). Research by Robic (1984) showed that the critical concentration of nitrate nitrogen in soil for lettuce is around 50 mg/kg. Furthermore, several studies have indicated that the form of nitrogen in applied fertilizers can significantly influence the level of nitrate accumulation in lettuce leaves (Bošković-Rakočević et al., 2009; El-Bassyouni, 2016; Bošković-Rakočević et al., 2023).

Some studies have also demonstrated a significant influence of ecological factors on the growth, yield, and quality traits of lettuce heads (Radovich et al., 2005). The impact of mineral fertilizers, particularly nitrogen, on morphological and agronomic traits—such as fresh weight, rosette diameter, root length, plant height, and number of leaves—has been confirmed in the studies by M'hamdi et al. (2014), Bhatta (2022), and Bošković-Rakočević et al. (2023).

Given that lettuce is a leafy vegetable widely consumed in the human diet, it is notable that relatively limited data are available on the effects of nitrogen and its various mineral forms on the growth and quality of lettuce cultivated in protected environments. In fact, it is essential to find a balance in the application of nitrogen fertilizers—so as not to compromise the yield potential of the cultivar, while simultaneously preventing excessive nitrate accumulation in the leaves, thus compromising the health benefits associated with this crop.

This study aimed to examine the effects of different formulations of nitrogen mineral fertilizers on vitamin C content, total nitrate concentration, head height, and fresh weight in five butterhead lettuce cultivars commonly grown in protected environments in Serbia.

## MATERIALS AND METHODS

The experiment was conducted during the winter-spring production season in a greenhouse located in Kalenić, Republic of Serbia. Five head-forming lettuce cultivars were selected for the study: Genezis 104, Zeralda, Majska kraljica, Atrakcija, and Nansen. These cultivars represent the most commonly grown butterhead lettuce types in greenhouses across Serbia.

The chemical analysis of the greenhouse soil where the experiment was conducted showed the following values: pH value (7.54), humus content (2.54%), total phosphorus (39.37 mg/100 g of soil), total potassium (11.31 mg/100 g of soil), and total nitrogen content (0.128%).

The trial was established following the standard methodology for lettuce cultivation in greenhouses, using a randomized complete block design with three replications. The size of each basic plot was 3 m<sup>2</sup>. Seeds for seedling production were sown in styrofoam trays with 105 cells, filled with "Klasmann" substrate. The 33-day-old seedlings were transplanted into the greenhouse on April 3, 2023. The planting layout was 25 × 30 cm, on black polyethylene mulch, with a drip irrigation system. Each plot contained 30 lettuce plants.

The experiment involved the application of different nitrogen mineral fertilizers with the following formulations: ammonium sulfate (21% N), ammonium nitrate (34% N), and urea (46% N). The applied nitrogen dose was 100 kg/ha for all tested formulations. Lettuce was harvested at the technological maturity stage on May 18, 2023. At that stage, the following parameters were measured: head weight, head height, and the total content of vitamin C and nitrates in the leaves of the five analyzed cultivars.

The chemical analysis of the soil included the determination of pH value, which was measured potentiometrically in a soil/KCl suspension (1:2.5). Humus content was determined using the Kotzmann method, while available phosphorus and potassium were determined using the AL-method according to Egner-Riehm (phosphorus by colorimetry, potassium by flame photometry).

The total vitamin C content in lettuce leaves was determined by Tillman's titrimetric method (Santos et al., 2016). The total phenolic content was assessed using the Folin-Ciocalteu method (Singleton et al., 1999), while total antioxidant activity was measured spectrophotometrically using the DPPH method (Xu et al., 2010). Nitrate content in the plant material was determined by the salicylic acid method (SM method) according to Cataldo et al. (1975).

Statistical analysis of the results was performed using a two-factor ANOVA model with the software Statistica 12. The significance of differences between means was determined using the LSD test (Least Significant Difference) for the factors cultivar, fertilization, and their interaction.

## RESULTS AND DISCUSSION

### *Fresh weight of lettuce heads*

The fresh weight of individual lettuce heads among the five tested cultivars in the experiment ranged from 145.3 g in the Nansen cultivar to 407.2 g in the Genezis cultivar. Statistical analysis showed that the differences in head weight among the analyzed cultivars were statistically significant (Table 1). The fresh weight of lettuce heads depends on the genetic characteristics of the cultivar, as well as on the type and application of fertilizers during plant cultivation (Ghimire et al., 2019; Ibrahim et al., 2019; Bošković-Rakočević et al., 2023).

The application of different nitrogen mineral fertilizers in this experiment had a significant impact on increasing the average head weight of lettuce. Treatments with calcium ammonium nitrate (CAN) and ammonium sulfate were particularly effective, while urea did not significantly affect head weight compared to the control (Table 1). These findings confirm the results reported by Soylemez (2021), who emphasized that proper and targeted nitrogen fertilization plays a key role in achieving high lettuce yields. A similarly significant effect of mineral nitrogen fertilizers on head weight and total yield was also observed in the studies by M'hamdi et al. (2014), Ibrahim et al. (2019), and Bewuket and Shewaye (2020).

**Table 1.** Analysis of variance for the effects of cultivar, fertilizer type, and their interaction on head weight, head height, total vitamin C content, and nitrate content in lettuce

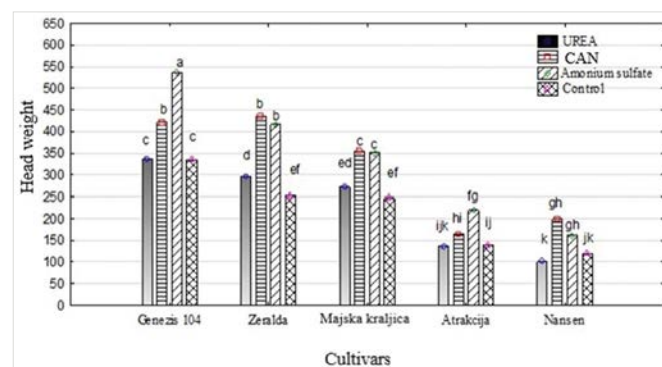
Traits/Treatments	Head weight (g)	Head height (cm)	Total vitamin C content (mg/100 g fresh weight)	Total nitrate content (mg/kg fresh weight)
Nitrogen fertilizers				
Urea	228.2 <sup>c</sup>	10.20 <sup>b</sup>	22.93 <sup>a</sup>	2.21 <sup>c</sup>
Calcium ammonium nitrate (CAN)	314.9 <sup>b</sup>	10.31 <sup>b</sup>	21.11 <sup>ab</sup>	3.59 <sup>a</sup>
Ammonium sulfate	336.7 <sup>a</sup>	12.51 <sup>a</sup>	22.33 <sup>a</sup>	3.11 <sup>b</sup>
Control	219.0 <sup>c</sup>	12.91 <sup>a</sup>	18.78 <sup>b</sup>	0.21 <sup>d</sup>
Cultivars				
Genezis	407.2 <sup>a</sup>	10.25 <sup>b</sup>	18.87 <sup>b</sup>	2.51 <sup>b</sup>
Zeralda	350.0 <sup>b</sup>	11.11 <sup>ab</sup>	20.22 <sup>ab</sup>	1.84 <sup>d</sup>
Majska kraljica	306.2 <sup>c</sup>	11.99 <sup>a</sup>	22.11 <sup>a</sup>	3.26 <sup>a</sup>
Atrakcija	164.9 <sup>d</sup>	12.02 <sup>a</sup>	22.43 <sup>a</sup>	2.21 <sup>c</sup>
Nansen	145.3 <sup>e</sup>	12.05 <sup>a</sup>	22.81 <sup>a</sup>	1.60 <sup>e</sup>
ANOVA				
Fertilizer	**	*	*	*
Cultivar	*	**	*	*
Fertilizer × Cultivar	*	*	*	*

The values denoted with different small letters within columns for every cut are significantly different ( $P < 0.05$ ) in accordance with the LSD test

Furthermore, the results of our study are consistent with the findings of Bošković-Rakočević et al. (2023), who confirmed the importance of selecting appropriate nitrogen fertilizers to ensure optimal growth and yield in different lettuce cultivars.

The interaction between cultivar and nitrogen fertilizer type revealed specific responses of individual cultivars to different fertilizer formulations. The cultivars Genezis, Nansen, and Majska kraljica responded significantly to treatments with calcium ammonium nitrate (CAN) and ammonium sulfate, while the effect of urea in these cultivars was similar to that of the unfertilized control. In the Zeralda cultivar, both CAN and ammonium sulfate also led to an increase in head weight, but in this case, a statistically significant difference was observed between urea and the control as well. For the cultivar Atrakcija, only ammonium sulfate had a significant effect,

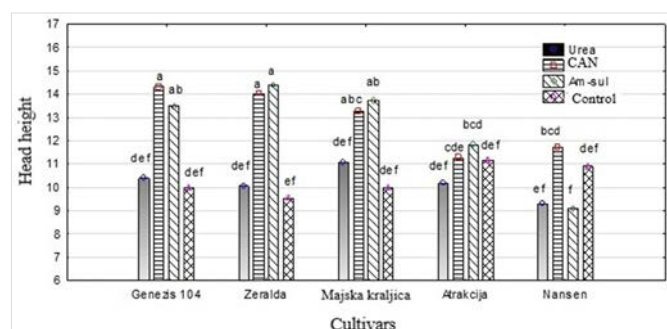
while the other formulations were at the level of the control (Figure 2). Similar patterns of interaction between cultivars and nitrogen fertilizers have been confirmed in the studies by M'hamdi et al. (2014) and Bošković-Rakočević et al. (2023).

**Figure 1.** Cultivar × nitrogen fertilizer interaction for lettuce head weight

### Head height

In this experiment, the head height of lettuce ranged from 10.25 cm for the Genezis cultivar to 12.05 cm for the Nansen cultivar. Statistically significant differences in plant height were observed only for the Genezis cultivar in comparison to the other analyzed cultivars, while the remaining cultivars did not differ significantly from each other in this trait (Table 1). The influence of cultivar on plant height and overall plant growth, as well as other parameters of lettuce, has been confirmed in studies by M'hamdi et al. (2014) and Ghimire et al. (2019). Sanni et al. (2016) also reported that plant height in lettuce is influenced by genetic factors, but the expression of these traits depends directly on soil fertility.

In this experiment, it was found that different nitrogen fertilizers had a significant effect on lettuce head height. The greatest increase in height was observed with urea and calcium ammonium nitrate (CAN), while ammonium sulfate and the unfertilized control resulted in considerably lower values (Table 1). Similar findings were reported by Rameshwar et al. (2022), who emphasized that fertilizers with higher nitrogen content promote greater plant elongation, whereas plants grown without nitrogen fertilizers exhibited the lowest head height. The positive and statistically significant influence of nitrogen fertilizers on the vegetative growth of lettuce was also confirmed in studies by Hasan et al. (2017), Ibrahim et al. (2019), and Bewuket and Shewaye (2020).



**Figure 2.** Cultivar × nitrogen fertilizer interaction for lettuce head height

The analysis of the interaction between cultivar and nitrogen fertilizer formulation showed that the cultivars Genezis, Zeralda, and Majska kraljica exhibited similar

behavior, with treatments involving CAN (calcium ammonium nitrate) and ammonium sulfate leading to significant differences in plant height. In the cultivar Atrakcija, no significant effect of fertilizer on plant height was observed, whereas in the cultivar Nansen, the dominant effect on height was recorded with the CAN formulation (Figure 2). Similar results regarding the interaction between cultivars and nitrogen fertilizers were reported by Ghimire et al. (2019), while Ibrahim et al. (2019) did not find a statistically significant interaction between cultivar and nitrogen fertilizer in relation to lettuce plant height.

### Vitamin C

This study revealed a statistically significant difference in vitamin C content among the five examined lettuce cultivars. The highest vitamin C content was recorded in the cultivar Nansen (23.55 mg/100 g), followed by Majska kraljica (22.69 mg/100 g). In contrast, the lowest values were observed in the cultivars Genezis (18.88 mg/100 g) and Zeralda (20.06 mg/100 g) (Table 1). Similar differences in vitamin C content among various lettuce genotypes have been reported in previous studies (Llorach et al., 2008; Govedarica-Lučić and Perković, 2015; Bošković-Rakočević et al., 2023), highlighting the significant role of genetic background in determining vitamin C levels. Additionally, many authors emphasize that external factors, such as agroecological conditions (Tuncay et al., 2011) and light intensity (Khan et al., 2018), may strongly influence the synthesis and accumulation of this vitamin.

Vitamin C is one of the key antioxidants in plant tissue, and its presence in leafy vegetables is of great importance. In addition to contributing to the plant's antioxidant defense, its nutritional value is particularly relevant for human health. It has been shown that vitamin C can reduce the harmful effects of nitrate decomposition products in the body, including the formation of methemoglobin (Kastori and Petrović, 2003; McKnight et al., 1999). Therefore, the favorable balance between vitamin C and nitrate content in fresh-consumed vegetables, such as lettuce, is of special importance.

Fertilization with different nitrogen formulations led to a statistically significant increase in vitamin C content compared to the unfertilized control. The significant influence of mineral nitrogen fertilizers on vitamin C content has also been confirmed in studies by El-Bassyouni (2016), Bošković-Rakočević et al. (2023), and in experiments involving nitrogen from various sources (Tuncay et al., 2011; Bhatta, 2022). The interaction between genotype and fertilization indicated different responses of individual cultivars to the applied nitrogen formulations, which was statistically significant (Table 1).

The interaction between cultivar and the application of mineral nitrogen fertilizers showed significant differences in vitamin C content among certain cultivars. In the Zeralda cultivar, a statistically significant difference in vitamin C content was observed between the control (no fertilization) and all treated variants with different nitrogen fertilizers, while no significant differences were recorded among the fertilizers themselves. A similar pattern was observed in the cultivar Majska kraljica, but in this case, the application of calcium ammonium nitrate (CAN) resulted in a statistically significant difference not only compared to the control, but also to the other formulations (urea and ammonium sulfate). In the cultivar Atrakcija, all fertilizer treatments (except for the control, which had the lowest vitamin C content) led to a statistically significant increase in this parameter.

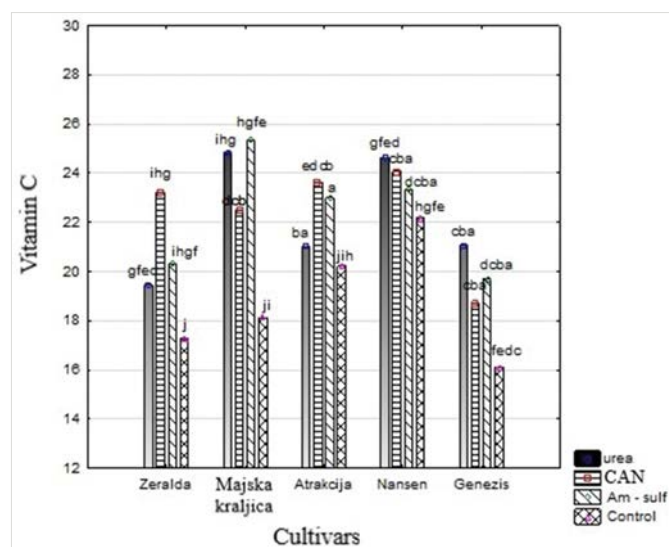


Figure 3. Cultivar × nitrogen fertilizer interaction for vitamin C content in lettuce leaves

In the cultivars Nansen and Genezis, significant differences were present only between the control and the treated variants, whereas no statistically significant differences were observed among the various fertilizer types in terms of vitamin C content (Figure 3).

The literature provides somewhat conflicting results regarding the effect of cultivar × fertilizer interaction on vitamin C content; while some authors have confirmed the significance of this interaction (Khan et al., 2018), others have not observed statistically significant effects (Bošković-Rakočević et al., 2023).

### Nitrate content

In this experiment, significant differences in nitrate content were observed among the five tested lettuce cultivars. The highest nitrate level was recorded in the cultivar Majska kraljica (3.26 mg/kg), while the lowest was found in Nansen (1.60 mg/kg). The remaining cultivars had intermediate values (Table 1). These differences confirm the importance of genetic factors in nitrate accumulation in plants, which is consistent with the findings of previous researchers (Catherine et al., 2017; Bošković-Rakočević et al., 2023). Similar results were reported by Xu et al. (2010) and Liu et al. (2014), while EFSA (2008) emphasized that nitrate content in leafy vegetables can vary greatly depending on cultivar, growing conditions, and harvest stage.

The application of different nitrogen mineral fertilizers also significantly affected nitrate content in the leaves. The highest increase in nitrate levels was recorded in the treatment with calcium ammonium nitrate (CAN), with an average value of 3.59 mg/kg, while fertilization with ammonium sulfate and urea resulted in slightly lower, yet still elevated levels compared to the unfertilized control (Table 1). These results support previous studies indicating that the type of nitrogen fertilizer has a significant influence on nitrate accumulation in leafy vegetables (El-Bassyouni, 2016; Khan et al., 2018; Bošković-Rakočević et al., 2023).

Urea, as an amide form of nitrogen, undergoes a slower transformation process in the plant (first to am-

monium, then to nitrate), allowing for more gradual nitrogen uptake and reducing the risk of excessive nitrate accumulation. In contrast, the application of CAN and ammonium sulfate results in faster nitrogen availability, particularly in nitrate form, which often leads to increased nitrate concentrations in plant tissues, especially when these forms are applied during the later stages of vegetation or at high doses (Ahmed et al., 2020; Mahmud et al., 2021).

The interaction between cultivar and fertilizer type was statistically significant, with cultivars responding differently to the application of individual formulations. The Genezis cultivar exhibited a significant increase in nitrate content under all fertilizer treatments compared to the control. In the Zeralda cultivar, significant differences were observed between the control and the treated variants, but without statistically significant differences among the fertilizers themselves. The cultivar Majska kraljica responded particularly to treatments with CAN and ammonium sulfate, showing higher nitrate content compared to the other variants. The cultivars Atrakcija and Nansen displayed similar response patterns, with significant differences relative to the control, but without significant differences among the individual treatments (Figure 4).

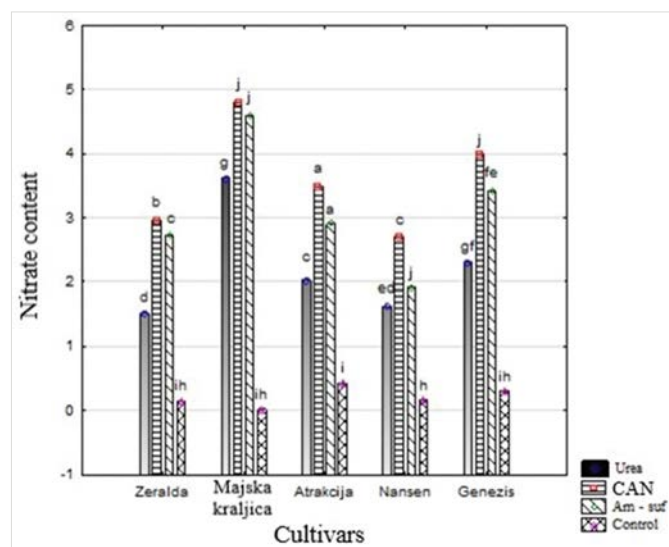


Figure 4. Cultivar × nitrogen fertilizer interaction for nitrate content in lettuce leaves

Similar findings confirming the existence of interactions between cultivar and nitrogen fertilizer were reported in studies by Khan et al. (2018), Bošković-Rakočević et al. (2023), and Bhatta et al. (2022), while some authors, such as Ibrahim et al. (2019), did not observe significant interactions. These results highlight the importance of carefully selecting the type of fertilizer in lettuce production in order to reduce nitrate content in the final product, in accordance with the recommended threshold levels (EFSA, 2008).

## CONCLUSION

This study examined the effects of different nitrogen fertilizer formulations on the morphological and phytochemical characteristics of five butterhead lettuce cultivars grown in a protected environment. The results showed significant differences among cultivars in terms of head weight, head height, vitamin C content, and nitrate accumulation. 'Genezis' and 'Majska kraljica' produced the heaviest heads but also exhibited higher nitrate levels, while 'Nansen' and 'Zeralda' showed lower nitrate content and higher vitamin C levels, indicating genotypic differences in nutrient accumulation and metabolism.

Among the tested fertilizers, calcium ammonium nitrate (CAN) and ammonium sulfate significantly improved head weight and contributed to higher nitrate content, while urea showed a milder effect. All nitrogen treatments increased vitamin C content compared to the control, highlighting the positive impact of nitrogen fertilization on nutritional quality. A significant interaction between cultivar and fertilizer type was observed, suggesting that genotypic responses to nitrogen formulations vary and should be taken into account when optimizing lettuce production.

These findings emphasize the importance of selecting appropriate cultivar-fertilizer combinations to achieve both high yield and desirable nutritional quality, while minimizing nitrate accumulation and ensuring food safety in greenhouse-grown lettuce.

## ACKNOWLEDGEMENT

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