Improving Yield and Quality-Related Physiological Characteristics of Lettuce by Integrated Inorganic and Organic Fertilizers Management

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Summary

The research aims to obtain more information on the nitrogen metabolism and nutraceutical properties of two types *Lactuca sativa* L. var. *crispa* L. type Batavia cv. 'Maritima' and type Lollo Rosso cv. 'Tuska' and one type *Lactuca sativa* L. var. *capitata* L. cv. 'Winter butterhead' under the implementation of different organic fertilizers. In this respect, the main goal of our study is to define a proper macro- and micronutrients source which has the most beneficial effect on yield as well as on various quality parameters of lettuce. The experiment was conducted in an unheated greenhouse in two consecutive seasons (2018/2019 and 2019/2020) under all the requirements for organic production. Four organic fertilizers (Italpollina", "Arcobaleno", "Lumbrical" and "EKOprop" were studied. From obtained results, it could be concluded that the impact of fertilizers on lettuce growth and quality has possessed cultivar dependence. The fertilizing with natural fertilizers, so that the nutrients they possess are strictly comprised of animal-based materials (dry bird manure "Italpolina", mix from bovine, horse and chicken manure "Arkobaleno") or compost (lumbricompost made by Red Californian worms "Lumbrical") of the three lettuce cultivars resulted in lower plant FW and yield, compared with NPK fertilizing. Only fertilizing with a mix of different beneficial microorganisms leads to the same plant FW and yield as fertilizing with inorganic fertilizer. Based on the higher plant FW and yield of all three lettuce cultivars the treatment with the fertilizer composed with beneficial microorganisms can be substituted with the treatment with inorganic NPC fertilizer. "EKOprop" fertilizing showed the lowest leaf nitrate concentration which correlated with higher activity of nitrogen assimilating enzyme nitrate reductase.

Key words

nitrate reductase, glutamine synthetase, *Lactuca sativa* L. var. *crispa* L.; *Lactuca sativa* L. var. *capitata* L., organic fertilizers

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Received: January 25, 2021 | Accepted: March 23, 2021

Introduction

Lettuce (*Lactuca sativa* L.) is one of the most commonly grown vegetables worldwide. It possesses a great reserve of antioxidants including vitamins A and C (Jimenez-Aguilar and Grusak, 2015). Lettuce is known for its anti-tumour properties and protection against cardiovascular diseases (Nicolle et al., 2004; Kim et al., 2016). Different lettuce varieties differ in their nitrogen (N), phosphorus (P) and potassium (K) requirements. Lettuces have a big and fast-growing leaf area which requires sufficient and easily assimilated nutrient elements. Farmers often use nitrogen fertilizers in order to increase yield and consequently many vegetables and forage plants accumulate great amounts of nitrates.

The use of organic fertilizers is important for yield improvement through constantly supplying the plant with micro and macronutrients. In addition, it improves the soil quality and fertility by increasing the level of soil organic matter, slowly releases significant amounts of nitrogen and phosphorus and increases the soil water-holding capacity (Muse, 1993). Organic fertilizers are naturally occurring materials of biological or mineral origin and are low in nutrient concentration or solubility, unlike chemical fertilizers that generally have high solubility and rapid availability (Barker et al., 2017). Manures are a source of almost all the essential nutrients for crop production, which content including nitrogen varies depending on the type of animal and feed ration, moisture content, storage, and handling methods (Van Averbeke and Yoganathan, 2003). The advantage of organic fertilizers is the ability to increase the amount of organic matter in the soil by improving soil physical, physicochemical, chemical and biological properties (Hole et al., 2005). Fertilizers of this type also act significantly more slowly than mineral fertilizers and thus plants are provided with long-term access to nutrients (Masarirambi et al., 2010). The active agents in organic fertilizers could be microorganisms that have the ability to prepare herbal assimilates and other biotic substances for the plant's needs. These fertilizers are recommended as an alternative or supplement for mineral nutrients.

Nitrogen requirements outline plant development which is also an indicator for plant responses to N nutrition. Nitrogen is important for the biosynthesis of amino acids, proteins, nucleic acids, chlorophyll, various primary and secondary metabolites, and hormones (Krapp, 2015). Most plants assimilate nitrogen in the form of nitrate, which is supplied in the form of inorganic and organic fertilizers (Temple et al., 1998). Plants also have the capacity to directly take up supplied ammonia. Plant nitrogen assimilation involves various steps. Once nitrate has been absorbed, it is transported to other parts of the plant. When nitrate has been transported to the cell, it is converted to nitrite by nitrate reductase (NR) in the cytosol (Beevers and Hageman, 1969). The produced nitrite is further reduced to ammonia by nitrite reductase in chloroplasts or plastids. The produced ammonia is assimilated to various pathways via the glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle (Krapp 2015). NR activity might be used as an indicator for plants ability to utilize nitrates as N supply (Vogel and Dawson, 1991). Most researches on NR activity are related to the implementation of various nitrate fertilizers. GS is a key enzyme in NH_4^+ assimilation. It catalyzes ATP-dependent fixation of the δ-carboxyl group of glutamate leading to the formation of glutamine, which consequently provides N groups, directly or via GOGAT, for the biosynthesis of other N-containing organic molecules. The metabolization of NH_4^+ into N-compounds is the chief mechanism of ammonium assimilation and detoxification in plants (Bernard and Habash, 2009).

Studies show that excessive use of fertilizers might lead to yield improvement but could simultaneously reduce the quality of crop production, due to over-accumulation of nitrates (Wang et al., 2002). This is a phenomenon, according to which the use of inorganic fertilizers increases lettuce yield but on the other hand, high amounts of nitrates accumulate in the leaves and decrease its quality. Nitrates in vegetables themselves are relatively harmless to humans, but their reaction products and metabolites lead to the accumulation of carcinogenic components (such as NO_2^-, NO and nitrosamines) which are harmful to human health (Salehzadeh et al., 2020). According to Commission Regulation (EU) No 1258/2011 for amending Regulation (EC) No 1881/2006 as regards maximum levels for nitrates in foodstuffs, the maximum level of nitrates in fresh lettuce (*Lactuca sativa* L.) harvested from 1 October to 31 March and grown under cover is 5000 mg NO_3^- kg^{-1}. According to Koudela and Petrikova (2008) the nitrate content in the lettuce leaves has shown the season of growing and the type of cultivar dependence. The authors recorded the statistically highest content of nitrates in type Lollo Rosso cultivated in the springtime and cultivar Dubáček cultivated in the autumn, when the light is lower in comparison with summertime when the light is higher.

Given the fact that lettuce growth is influenced by the various fertilizers addition, this study aimed to determine which cultivar would give a satisfactory yield with increased beneficial qualities for the consumer's health. The optimization of lettuce production would imply that its tolerance towards changing environmental conditions is guaranteed as well as an assessment of the impact of NR and GS activity on nitrate accumulation in lettuce grown on different farming substrates is performed. Soil is a basic anchor to support plant growth, and when properly fertilized, gives a meaningful crop yield. In this respect, the main goal of this study is to define the source of different organic fertilizers which have the most beneficial effect on yield as well as on various quality parameters of lettuce.

Material and Methods

The effect of different fertilizers on growth, nitrogen metabolism and photosynthetic pigments content of three lettuce cultivars was studied (two types leaf lettuce (*Lactuca sativa* L. var. *crispa* L.) type Batavia cv. 'Maritima' and type Lollo Rosso cv. 'Tuska'; one type head lettuce (*Lactuca sativa* L. var. *capitata* L.) cv. 'Winter butterhead').

The lettuce was grown for two years (2018/2019 and 2019/2020) in the plastic unheated greenhouses in the experimental field of the Agricultural University – Plovdiv. The bio seeds were sown on a seedling mixture (80% Perlite: 20% Lumbricompost, v/v) in October in containers with trays with 150 pots. A total of 3,000 plants (1,000 plants of each cultivar) were planted in phase 4-5 leaf in November on alluvial meadow soil (mollic fluvisol, FAO 2006) in a greenhouse. Soil agrochemical characteristics: pH (KCl) 7.40, total N 55.3 mg kg^{-1}, NH_4^+ + NO_3^- = 41.5 + 13.8 = 55.3 mg kg^{-1}, mobile phosphorus (P_2O_5) 22.00 mg 100 g^{-1}, mobile potassium

(K_2O) 52.50 mg 100 g^{-1}. The experiment was based on the block method in four repetitions in 28 plants per repetition, with a plot size of 3.36 m^2. The experiment was conducted with the application of complete technology for organic production according to the requirements of Council Regulation (EC) No 834/2007. Irrigation was carried out with a drip system. Plant protection was applied two times with Neemazal T/S and two times with Funguran OH 50 WP during the vegetation. The hydrothermal conditions for the period from planting the lettuces to their consumer maturity are given in Table 1.

Two organic fertilizers ("Italpollina" and "Lumbrical") already used in organic vegetable production and two less-known but promising fertilizers ("Arkobaleno" and "EKOprop") were used (Table 2).

Six treatments were compared: C – control, non fertilized plants; NPK - plants, fertilized with N:P:K=1:2:1; I - plants, fertilized with "Italpollina"; A - plants, fertilized with "Arcobaleno"; L - plants, fertilized with "Lumbrical"; E - plants, fertilized with "EKOprop". The granular fertilizers were introduced as the main fertilizer with broadcasting with pre-planting tillage in the following norms: N – 125 kg ha^{-1}, P_2O_5 – 12.5 kg ha^{-1}, K_2O – 47.5 kg ha^{-1}, "Italpollina" – 250 kg ha^{-1}, "Arcobaleno" - 1000 kg ha^{-1}, "Lumbrical" 4000 L ha^{-1}, "Ekoprop" (is applied by double treatment in the seedling phase and after planting) 1 kg/ha. The growth of the plants was monitored by making three measurements - from the beginning of harvesting every 7 days. The yield is determined as the product of the mass of plants and the number of plants per hectare.

The extraction medium for determination of nitrate reductase (NR: NADH, EC 1.6.6.2) and glutamine synthetase (GS, EC 6.3.1.2) in plant tissue (0.5 g fresh leaf material) contained 50 mM Tris-HCl (pH 8.0), 1 μM Na_2MoO_4, 10 mM $MgSO_4{\cdot}7H_2O$, 1 mM EDTA, 10 mM L-cysteine, 1% PVP-40, 1 g Dowex (Frechilla et al., 2000). The extract was filtered through one layer of cheesecloth, centrifuged at 10 000g for 20 min (4 °C), and the supernatant was used for the enzyme activity assays. NR activity was measured according to Hageman and Reed (1980). GS activity was determined by O' Neal and Joy (1973). Protein content was determined as in Bradford (1976) with BSA as a standard. Nitrate content was determined according to Zhao and Wang (2017). Total chlorophyll and carotenoids in the lettuce leaves were extracted in 80% acetone. The pigments were determined spectrophotometrically as described by Lichtenthaler (1987). Reducing sugars were analyzed by the phenol-sulphuric acid procedure by Ashwell (1966).

Statistical Analysis

The data were statistically processed by analysis of variance (ANOVA) for comparison of means, and significant differences were calculated according to Fisher's least significance difference (LSD) test at the $P < 0.05$ significance level using a statistical software package (Statgraphics Plus, version 5.1 for Windows, Statistical Graphics Corporation; Warrenton, VA, USA).

Table 1. Hydrothermal conditions in the region of Plovdiv during the period of the experiment

Months Years	Av.t (°C)	δ	Max. (°C)	date	Min.t. (°C)	Date	Σ mm	%
October 2018	13,9	2,2	27	8	-0,8	26	5,2	13
October 2019	14,4	2	30,5	1	0,0	29	36	65
November 2018	7,3	0,5	27,4	2	-1,5	30	53	84
November 2019	10,8	3,8	23	5	2,8	30	72	164
December 2018	2,9	0,5	16,5	23	-6,6	1	35	70
December 2019	4,4	2	17	15	-6,5	6	25	58
January 2019	2,6	2,3	14,8	17	-8,6	9	31	77
January 2020	3,5	3,2	17	28	-10,5	8	3	8
February 2019	4,7	1,9	20,0	20	-11,2	24	17,2	50
February 2020	6,4	3,6	21,5	1	-7	9	51	149
March 2019	10,6	3,8	27	17	-3.2	5	9	22
March 2020	8,9	2,1	23,8	13	-4	17	94	228
April 2019	12,6	0,4	27	26	-1,2	3	61	145
April 2020	11,5	-0,7	27,8	18	-2,5	8	90	215

Table 2. Physicochemical characteristics of tested organic fertilizers

Trade name	Composition	Ingredients
Italpollina (Italpollina S.p.a, Località Casalmenini, 10 37010 - Rivoli Veronese (VR), Italy)	Dry bird manure	total N “4%”, phosphorus “P_2O_5 4%”, potassium “K_2O 4%”, water soluble magnesium “MgO 0,5%”, water soluble Fe “0,8%”, water soluble B “0,2%”, organic carbon “41%”, organic matter “70,7%”, humic acids “5%”, fulvic acids “12%”, pH 7.
Arcobaleno (Agrofertil, Società Cooperativa Agricola - Santa Sofia, Forese Macallè str., Italy)	Mix from bovine, horse and chicken manure	organic N “4-5%”, phosphorus “P_2O_5 3,5%”, potassium “K_2O 3.5%”, calcium “CaO 5-8%”, water soluble magnesium “MgO 0,8-1%”, organic carbon “30%”, organic matter 55-60%”, Fe 0.31-0.32%, pH(H_2O) 6-8.
Lumbrical - (Bogaq EOOD farm for organic fertilizers production, village Kostievo, Bulgaria).	lumbricompost made by Red Californian worms (*Lumbricus rubelus*)	total N “1.71%”, water soluble N-NH_4 – 1.75 mg/kg, water soluble N-NO_3 - 804 mg/kg, phosphorus “P_2O_5 3.49%”, potassium “K_2O 1.71%”, calcium “CaO 6.25%”, water soluble magnesium “MgO 2.14%”, organic carbon “27-35%”, dry matter “44.2%”, organic matter “47.24%”, pH(H_2O) 7.85
EKOprop® NX	mix of different microorganisms	Mycorrhizae (*Glumos* spp) 1%, Rhizobacteria (*Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp.) 2,3 x 10^5 CFU g^{-1}, Fungi (*Arthrobothrys, Paecilomyces, Monacrosporium, Myrothecium*) 1,0 x 10^6 CFU g^{-1}, Trichoderma (*Trichoderma* spp.) 5,0 x 10^5 CFU g^{-1}.

Results and Discussion

Effect of Different Fertilizers on Lettuce Growth and Quality

Balanced fertilization of N, P, K and Ca as well as microelement addition is of great importance for lettuce quality and yield. In these experiments, the effect of different fertilizers, organic and inorganic NPK fertilizers was evaluated over three cultivars of lettuce grown in unheated greenhouses (two types of leaf lettuce (*Lactuca sativa* L. var. *crispa* L.) type Batavia cv. 'Maritima' and type Lollo Rosso cv. 'Tuska'; one type head lettuce (*Lactuca sativa* L. var. *capitata* L.) cv. 'Winter butterhead'), on fresh and dry biomass production, pigment content and changes in nitrogen metabolism. Fertilization with organic and chemical fertilizers influenced differently the lettuce growing parameters (Table 3). The largest biomass was accumulated in lettuce cultivar 'Maritima', followed by 'Winter butterhead' and 'Tuska' at all studied fertilizers treatments. The increase of biomass 20-26% was observed in all three lettuce cultivars, grown on soil enriched with chemical fertilizers and with a mix of different microorganism “EKOprop” fertilizer in comparison with unfertilized plants. The smallest leaves number was measured in the lettuce cv. 'Tuska', followed by 'Maritima' and 'Winter butterhead' in all investigated variants. Regarding the number of leaves per plant, and rosette diameter, fertilizing with a mix of different microorganism “EKOprop” led to an increment in leaves number per plant, reaching the same number as in the variants fertilized with chemical NPK fertilizers.

In all three lettuce cultivars, inorganic and organic fertilization had a different impact on yield per area. Concerning yield kg/ha, again the best results were obtained in fertilization of all three lettuce varieties with a mix of different microorganism fertilizer “EKOprop”. Yields were comparable with those of plants grown on soil with chemical NPK fertilizers. Similar results were reported by Kim et al. (2016) in lettuce varieties Lollo Rosso and Iceberg. The yield (kg ha^{-1}) of lettuce cultivars 'Maritima' and 'Tuska' fertilized with “EKOprop” was comparable with the one from plants grown on chemical fertilized soil, while in 'Winter butterhead' the yield was higher. The relative yield was the highest when a mix of different microorganism fertilizer “EKOprop” was used for fertilization on lettuce cultivar „Winter butterhead” followed by 'Tuska', and 'Maritima'. Some authors concluded that chemical N fertilisation significantly increased lettuce fresh biomass, but the chicken, pigeon or buffalo manure application has led to a not statistically significant increase (El-Shinawy et al., 1999; Haruna, 2011). The losses of lettuce leaf biomass receiving organic manure solution may be due to the fact that organic manure realize available N mainly as NH_4^+ and little or none as NO_3, while the inorganic fertilizers realize mainly nitrate N (Smith and Hadley, 1989). Abd-Elmoneim et al. (1997) report that the nitrogen supplied in the form of nitrate is frequently much more effective on the growth than ammonium form. On the other hand, Masarirambi et al. (2010) recommend for farmers to use chicken manure instead of inorganic fertilizer to obtain higher yields of *Lactuca sativa* L. cv. 'Commander' with higher leaf number, area and biomass. Therefore, the impact of fertilizers on lettuce growth has possessed cultivar dependence.

Regarding reducing sugar, the highest content was found in lettuce cultivar „Tuska” followed by 'Maritima' and 'Winter butterhead' (Fig. 1). All three studied lettuce varieties showed an increase of leaf reducing sugar, induced not only by chemical fertilizer but also by organic fertilizers. We found the highest leaf reduced sugar increase when the soil was enriched with Lumbrical (33% in comparison with the control). According to Zhang et al. (2017), the use of organic fertilizer on lettuce growth leads to a decrease in the soluble sugars and chlorophyll content compared to the inorganic fertilizer.

Effect of Different Fertilizers on Physiological Characteristics

All three lettuce varieties grown on organically fertilized soil exhibited differences in NR activity, the key enzyme for the production of proteins in plants which reduces nitrate to nitrite (Fig. 2A). Nitrate content in lettuce leaves directly correlated with the activity of N-assimilating enzyme NR (Fig. 2A, C).

Table 3. Growth parameters of lettuce fertilized with inorganic and organic fertilizers

	Lactuca sativa L. var. *crispa* L., type Batavia cv. 'Maritima'	*Lactuca sativa* L. var. *crispa* L., type Lollo Rosso cv. 'Tuska'	*Lactuca sativa* L. var. *capitata* L., cv. 'Winter butterhead'
	FW (g plant^{-1})		
C	669.03±33.45a	254.06±12.70a	476.80±23.84a
NPK	799.94±40.00b	321.78±16.09b	516.22±25.81a
I	664.27±33.21a	266.03±13.30a	491.58±24.58a
A	657.08±32.85a	258.86±12.94a	480.47±24.02a
L	636.17±31.81a	255.05±12.75a	499.11±24.96a
E	775.04±38.75b	317.74±15.29b	568.22±28.41b
LSD	62.23	24.56	45.03
	Leaves (number plant^{-1})		
C	40.47±2.02ab	27.31±1.37a	42.52±2.13ab
NPK	45.22±2.26c	29.33±1.47ab	41.86±2.09a
I	38.94±1.95a	28.33±1.42ab	44.45±2.22ab
A	40.44±2.02ab	27.06±1.35a	43.42±2.17ab
L	39.08±1.95a	27.02±1.35a	45.80±2.29b
E	42.93±2.15bc	30.07±1.50b	45.93±2.30b
LSD	3.67	2.50	3.90
	Rosette diameter (cm)		
C	38.64±1.93a	33.39±1.67a	39.05±1.95a
NPK	40.82±2.04a	34.67±1.73a	38.58±1.93a
I	39.30±1.97a	33.84±1.69a	38.80±1.94a
A	39.60±1.98a	34.32±1.72a	39.08±1.95a
L	38.78±1.94a	34.54±1.73a	39.86±2.00a
E	40.20±2.01a	35.61±1.78a	39.48±1.97a
LSD	3.52	2.86	3.48
	Yield (kg ha^{-1})		
C	42646.89±2132.34a	15302.70±765.00a	29837.84±1491.76a
NPK	50992.17±2550.12b	19382.03±969.12b	30191.22±1510.05a
I	43122.64±2156.54a	16335.92±816.58a	29036.89±1452.11a
A	43039.23±2152.29a	16046.52±801.89a	30347.41±1516.56a
L	40922.90±2046.11a	15513.29±775.55a	29772.46±1488.46a
E	49562.66±2478.01b	20316.61±1016.16b	35450.72±1773.21b
LSD	4021.08	1535.39	2744.16
	Relative yield (%)		
C	83.63	78.95	98.83
NPK	100.00	100.00	100.00
I	84.57	84.28	96.18
A	84.40	82.79	100.52
L	80.25	80.04	98.61
E	97.18	104.82	117.42

Note: ". C – control, non fertilized plants; NPK - plants, fertilized with N:P:K=1:2:1; I - plants, fertilized with "Italpollina"; A - plants, fertilized with "Arcobaleno"; L - plants, fertilized with "Lumbrical"; E - plants, fertilized with "EKOprop". Values are means ± SE, n=4; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multi-factor analysis

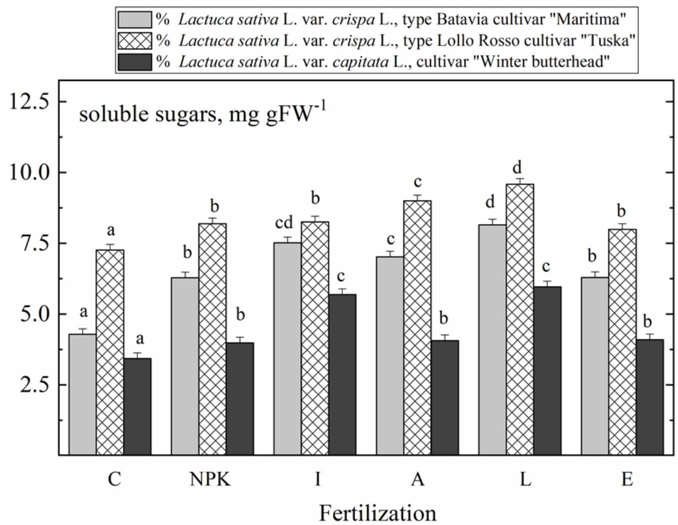

Figure 1. The soluble sugar content in the leaves of the three lettuce cvs. 'Maritima', 'Tuska' and 'Winter butterhead'

Note: C – control, non fertilized plants; NPK - plants, fertilized with N:P:K=1:2:1; I - plants, fertilized with "Italpollina"; A - plants, fertilized with "Arcobaleno"; L - plants, fertilized with "Lumbrical"; E - plants, fertilized with "EKOprop". Values are means ± SE, n=4; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multi-factor analysis

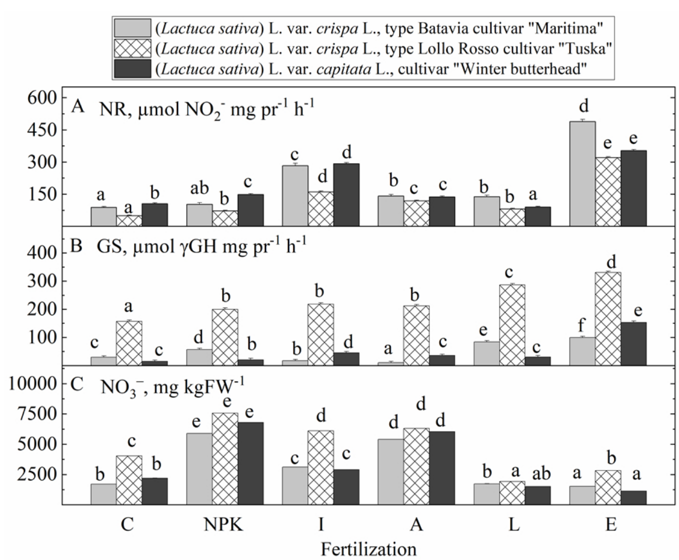

Figure 2. The activity of enzymes nitrate reductase (NR), glutamine synthetase (GS), and nitrate content in the leaves of the three lettuce cvs. 'Maritima', 'Tuska' and 'Winter butterhead'

Note: C – control, non fertilized plants; NPK - plants, fertilized with N:P:K=1:2:1; I - plants, fertilized with "Italpollina"; A - plants, fertilized with "Arcobaleno"; L - plants, fertilized with "Lumbrical"; E - plants, fertilized with "EKOprop". Values are means ± SE, n=4; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multi-factor analysis

The highest NR activity was detected in all three lettuce cultivars grown on soils enriched with a mix of different microorganism fertilizer "EKOprop", followed by dry bird manure Italpollina, where leaf nitrate content was the lowest. Fertilization of lettuce cvs. 'Maritima' and 'Tuska' with all four studied natural fertilizers led to an increase in the NR activity, with the highest increase (4 times) found in plants grown on soil fertilized with a mix of different microorganism "EKOprop" compared to untreated plants. The most significant increase in the GS activity (2 times) was found in the lettuce cv. 'Tuska' leaves, grown on soil with lumbricompost made by Red Californian worms "Lumbrical", where the lowest nitrate content was assessed (Fig. 2B). The content of nitrates in all three lettuce cultivars grown in inorganic NPK fertilized soil (5895, 7581 and 6800 mg NO_3^- kg^{-1}) and Arcobaleno" fertilized soil (5418, 6312, 6042 mg NO_3^- kg^{-1}) was higher than the maximum levels of nitrates (5000 mg NO_3^- kg^{-1}) in fresh lettuce harvested from 1 October to 31 March and grown under cover according to Commission Regulation for amending Regulation of maximum levels for nitrates in foodstuffs. The nitrate content was 5 times higher than control plants, which had the lowest NR activity as well. From the three studied lettuce varieties, the highest GS activity was measured in cv. 'Tuska'. The greatest increase in GS activity (1.5 times) was estimated in lettuce grown on lumbricompost made by Red Californian worms "Lumbrical" enriched soil compared with non-fertilized control. The application of different nitrogen fertilizer sources modifies the balance of the soil chemical properties and makes an impact on the yield and quality of lettuce and especially on the nitrates content (Simeonova et al., 2015). Lettuce accumulates the highest amounts of nitrates among all vegetables (Iammarino et al, 2014). Although meteorological conditions, the period of growth and cultivar are among the factors that influence nitrate accumulation, the greatest impact is attributed to N fertilization (Gonnella and Elia, 2000; Slipka et al., 2000). Stancheva et al. (2004) have estimated lower fresh weight values in lettuce grown on organic compared to mineral fertilizer. NR has a great influence on nitrate accumulation in plants because it is induced by nitrate and is a limiting factor for nitrate assimilation in higher plants. The relationship between NR activity and nitrate accumulation is dependent on the exogenous nitrate, as well as various factors, such as temperature, light, sucrose, nitrogen metabolites as amino acids and glutamine, and many others (Chen et al., 2004). It is insufficient to evaluate nitrate accumulation only by the NRA.

Regarding changes in pigment content in the cv. 'Winter butterhead' leaves, grown on soils enriched with inorganic fertilizers or the four considered organic fertilizers, an increase in chlorophyll and carotenoid content was observed in comparison with non-fertilized plants (Fig. 3). The smallest increase of pigment content was found in plants treated with "EKOprop". The highest carotenoid content (nearly 3 times higher) was assessed only in the leaves of plants grown on soil enriched with Italpollina. The total content of chlorophyll *a* and *b* in "Maritima" leaves was the highest in plants fertilized with a mix of different microorganism fertilizer "EKOprop" and control unfertilized plants. Soil adding of chemical fertilizers or natural organic fertilizers ("Italpollina" and "Arcobaleno"), led to a reduction of total chlorophyll content. Reduced pigment content was assessed in the leaves of „Tuska" treated with chemical fertilizer in comparison with non-fertilized plants.

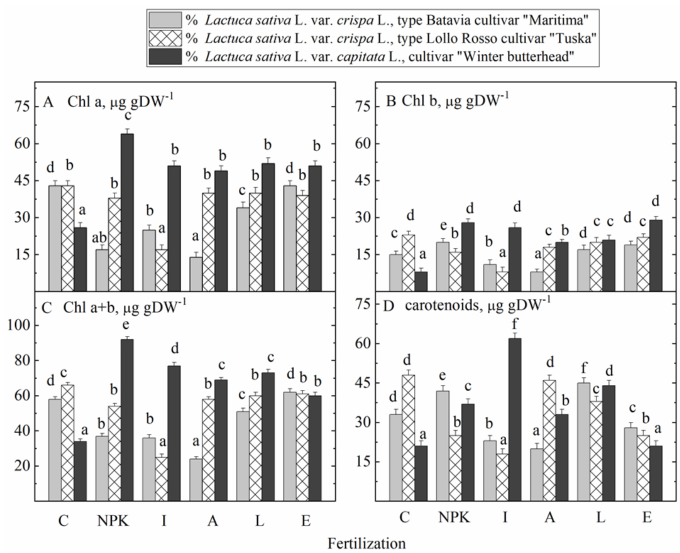

Figure 3. The Chlorophyll a, Chlorophyll b, Chlorophyll a+b, and carotenoids content in the leaves of the three lettuce cvs. 'Maritima', 'Tuska' and 'Winter butterhead'

Note: C – control, non fertilized plants; NPK - plants, fertilized with N:P:K=1:2:1; I - plants, fertilized with "Italpollina"; A - plants, fertilized with "Arcobaleno"; L - plants, fertilized with "Lumbrical"; E - plants, fertilized with "EKOprop". Values are means ± SE, n=4; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multi-factor analysis

The lettuce treated with organic fertilizers did not show significant changes in total chlorophyll content compared with the inorganic fertilized, only in "Italpollina"-treated plants the values were lowered. The higher chlorophyll content is usually associated with better N, Mg, Fe and Mn nutrition of plants, which leads to improved photosynthetic capacity. More than 75% of the total organic N in the plant is located in chloroplasts mainly in the form of enzymes and each deficiency has a direct impact on chlorophyll synthesis (Salisbury and Ross, 1992). Carotenoids are components of non-enzymatic defence system which act in detoxification of 1O2 and thus protect the photosynthetic machinery from damage (Edreva et al, 2004). This ability of carotenoids is probably due to their specific chemical structure as they contain a chain of isoprene residues connected with multiple conjugated double bonds, i.e. delocalize π electrons (Prakash and Gupta, 2014). Regarding carotenoids, the highest content was found in the cv. 'Winter butterhead' leaves, grown on soil enriched with "Italpollina", around 36% higher in comparison with organic fertilizers treated lettuce. Each increase or decrease of the chlorophyll a/b ratio could lead to damage to the reaction centres of the two photosystems. This would also impact yield and total antioxidant content.

Conclusion

In conclusion, it could be highlighted that in all three studied lettuce varieties fertilization with inorganic and organic fertilizers caused an increase in the NR and GS activities. The highest enzymes activity was estimated in the variants with the fertilizer composed of beneficial microorganisms "EKOprop", where leaf nitrate content was low. The content of reducing sugars in *Lactuca sativa* L. var. *crispa* L. type Lollo Rosso cv. 'Tuska' leaves when lettuce was treated with lumbricompost made by Red Californian worms "Lumbrical", was the highest. An increase in reducing sugars could benefit nitrogen metabolism and thus prevent nitrate accumulation. The search for appropriate N fertilizers is increasing to support growth and respectively increase the yield of economically important crop plants. For this reason, strategies are being developed aimed at yield improvement through the implementation of different fertilizers and towards clarification of mechanisms of action of biologically active substances and their impact on human health.

Acknowledgements

This study was conducted with financial support from the Centre of Research, Technology Transfer and Protection of Intellectual Property Rights at the Agricultural University-Plovdiv. The authors are thankful to Madlen Boychinova, Mariana Sichanova and Janet Mincheva for excellent technical assistance.

References

Amending Regulation (EC) No 1881/2006 as Regards Maximum Levels for Nitrates in Foodstuffs, (2011). COMMISSION REGULATION (EU) No 1258/2011 of 2 December 2011, Official Journal of the European Union, L 320, 15-17

Ashwell G. (1966). The Phenol-Sulphuric Acid Reaction for Carbohydrates. Method Enzymol 8, 93-95.

Barker A. V., Eaton T., Meagy M. J., Jahanzad E., Bryson, G. (2017). Variation of Mineral Nutrient Contents of Modern and Heirloom Cultivars of Cabbage in Different Regimes of Soil Fertility. J Plant Nutr. 40 (17): 2432–2439. doi: 10.1080/01904167.2017.1346682

Beevers L., Hageman R. H. (1969). Nitrate Reduction in Higher Plants. Ann Rev Plant Physiol. 20: 495-522. doi: 10.1146/annurev.pp.20.060169.002431

Bernard S. M., Habash D. Z. (2009). The Importance of Cytosolic Glutamine Synthetase in Nitrogen Assimilation and Recycling. New Phytol. 182 (3): 608-620. doi: 10.1111/j.1469-8137.2009.02823.x

Bradford M. (1976). A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Dinding. Anal Biochem. 72, 248-254. doi: 10.1006/abio.1976.9999

Edreva A., Georgieva I.D., Stoilova Ts., Cholakova N., Dagnon S. (2004). Biochemical and Histochemical Study of the Induced Resistance in Tomato- *Clavibacter michiganensis* ssp. *michiganensis* System. C R Acad Bulg Sci. 57 (9): 61-66.

EI-Shinawy M. Z., Abd-Elmoniem E. M., Abou-Hadid A. F. (1999). The Use of Organic Manure for Lettuce Plants Grown under NFTConditions. Acta Hortic. 486: 315-318. doi: 10.17660/ActaHortic.2001.559.80

Frechilla S., Gonzalez E. M., Royuela M., Minchin F. R., Aparicio-Tejo P. M. Arrese-Igor C. (2000). Source of Nitrogen Nutrition (Nitrogen Fixation or Nitrate Assimilation) Is a Major Factor Involved in Pea Response to Moderate Water Stress. J Plant Physiol. 157 (6): 609-17. doi: 10.1016/S0176-1617(00)80003-6

Chen B.-M., Wang Z.-H., Li S.-X., Wang G.-X., Song H.-X., Wang X.-N. (2004). Effects of Nitrate Supply on Plant Growth, Nitrate Accumulation, Metabolic Nitrate Concentration and Nitrate Reductase Activity in Three Leafy Vegetables. Plant Sci. 167 (3): 635-643. doi: 10.1016/j.plantsci.2004.05.015

Council Regulation (EC) No 834/2007 of 28 June 2007 on Organic Production and Labelling of Organic Products and Repealing Regulation (EEC) No 2092/91 *OJ L 189, 20.7.2007, p. 1–23 (BG, ES, CS, DA, DE, ET, EL, EN, FR, IT, LV, LT, HU, MT, NL, PL, PT, RO, SK, SL, FI, SV) Special edition in Croatian: Chapter 15 Volume 008 P. 139 – 161*

FAO, 2006, World Reference Base for Soil Resources, Rome, Italy, pp. 131

Gonnella M. G., Conversa Elia A. (2000). Nitrogen Level Yield and nitrate Accumulation in Soilless Grown Lettuce (*Lactuca sativa* L.) In: Proceeding of Scientific Meeting of Italian Horticulture Society, 28-30 May, 2000, Sirmone, Brescia, Italy, pp. 229-230

Hageman R. H., Reed A. J. (1980). Nitrate Reductase from Higher Plants. Methods Enzymol. 69: 270-280. doi: 10.1016/S0076-6879(80)69026-0

Haruna I. M. (2011). Growth and Yield of Sesame (*Sesamum indicum* L.) as Affected by Poultry Manure, Nitrogen and Phosphorus at Samaru, Nigeria. J Anim Plant Sci. 21 (4): 653-659.

Hole D. G., Perkins A. J., Wilson J. D., Alexander I. H., Grice P. V., Evans A. D. (2005). Does Organic Farming Benefit Biodiversity? Biol Conserv. 122 (1): 113-130. doi: 10.1016/j.biocon.2004.07.018

Iammarino M., Di Taranto A., Cristino M. (2014). Monitoring of Nitrites and Nitrate Levels in Leafy Vegetables (Spinach and Lettuce): A Contribution to Risk Assessment. J Sci Food Agr. 94 (4): 773-778. doi: 10.1002/jsfa.6439

Jimenez-Aguilar D. M., Grusak M. A. (2015). Evaluation of Minerals, Phytochemical Compounds and Antioxidant Activity of Mexican, Central American, and African Green Leafy Vegetables. Plant Foods Hum Nutr. 70: 357-364. doi: 10.1007/s11130-015-0512-7

Kim M.J., Moon Y., Tou J.C., Mou B., Waterland N.L. (2016). Nutritional Value, Bioactive Compounds and Health Benefits of Lettuce (*Lactuca sativa* L.). J. Food Compos Anal. 49: 19-34. doi: 10.1016/j.jfca.2016.03.004

Koudela M., Petříková K. (2008) Nutrients Content and Yield in Selected Cultivars of Leaf Lettuce (*Lactuca sativa* L. var. *crispa*). Hort Sci. (Prague) 35 (3): 99-106. doi: 10.17221/3/2008-HORTSCI

Krapp A. (2015). Plant Nitrogen Assimilation and Its Regulation: A Complex Puzzle with Missing Pieces. Curr Opin Plant Biol. 25: 115-122. doi: 10.1016/j.pbi.2015.05.010

Lichtenthaler H. K. (1987). Chlorophylls and Carotenoids, the Pigments of Photosynthetic Biomembranes. Methods Enzymol. 148, 350-382. doi: 10.1016/0076-6879(87)48036-1

Masarirambi M., Mduduzi M., Olusegun H., Oseni T., Sibiya T. E. (2010). Effects of Organic Fertilizers on Growth, Yield, Quality and Sensory Evaluation of Red lettuce (*Lactuca sativa* L.) 'Veneza Roxa'. Agric Biol J N Am. 1 (6), 1319-1324. doi: 10.5251/abjna.2010.1.6.1319.1324

Muse J. K. (1993). Inventory and Evaluation of Paper Mill By-Products for Land Application. MSc, Auburn University, Auburn, Alabama, USA.

Nicolle C., Cardinault N., Gueux E., Jaffrelo L., Rock E., Mazur A., Amouroux P., Remesy C. (2004). Health Effect of Vegetable-Based Diet: Lettuce Consumption Improves Cholesterol Metabolism and Antioxidant Status in the Rat. Clin Nutr. 23 (4): 605-614. doi: 10.1016/j.clnu.2003.10.009

O'Neal D., Joy K. (1973). Glutamine Synthetase of Pea Leaves, I. Purification, Stabilization and pH Optima. Arch Biochem Biophys. 159 (1), 113-122. doi: 10.1016/0003-9861(73)90435-9

Prakash D, Gupta C. (2014). Carotenoids: Chemistry and Health Benefits CAB International. Chapter 12, In: Phytochemicals of Nutraceutical Importance (Prakash D., Sharma G., eds)., CABI Publishing, pp. 181-195. doi: 10.1079/9781780643632.0000

Salehzadeh H., Maleki A., Shahmoradi B. (2020). The Nitrate Content of Fresh and Cooked Vegetables and Their Health-Related Risks. PLoS ONE 15 (1): e0227551. doi: 10.1371/journal.pone.0227551

Salisbury F. B., Ross C. (1992). Plant Physiology. Wadsworth Publishing Company, Belmont, Califonia 94002, a division of Wadsworth, Inc.

Simeonova R., Bratkov V. M., Kondeva-Burdina M., Vitcheva V., Manov V., Krasteva I. (2015). Experimental Liver Protection of n-Butanolic Extract of *Astragalus monspessulanus* L. on Carbon Tetrachloride Model of Toxicity in Rat Redox Report Commun Free Radic Res. 20 (4): 45-153. doi: 10.1179/1351000214Y.0000000115

Slipka Y., Pribylova P., Kolarova S. (2000). The Accumulation of Nitrates in Different Parts of Lettuce (*Lactuca sativa* L.). Ser Crop Res (Czech Republic). 17 (2): 111-120.

Smith S. R., Hadley P. (1989). A Comparison of Organic and Inorganic Nitrogen Fertilizers: Their Nitrate-N and Ammonium-N Release Characteristics and Effects on the Growth Response of Lettuce (*Lactuca sativa* L. cv. *Fortune*). Plant Soil 115: 135-144. doi: 10.1007/BF02220704

Stancheva I., Mitova I., Atanasova E., Toncheva R. (2004). Influence of the Sources and Norms of Nitrogen Fertilization on the Yield and Quality of Lettuce. Ecol Ind 6 (1): 82-83.

Statgraphics plus Software Version 5.1. (1994) Statistical Graphics Corporation, Warrenton, VA

Temple S. J., Vance C. P., Gantt J. S. (1998). Glutamate Synthase and Nitrogen Assimilation. Trends Plant Sci. 3 (2): 51-56. doi: 10.1016/S1360-1385(97)01159-X

Van Averbeke A., Yoganathan S. (2003). Using Kraal Manure as Fertilizer. Department of Agriculture, Pretoria, South Africa.

Vogel C. S., Dawson J. O. (1991). Nitrate Reductase Activity and Photosynthesis of Black Alder Exposed to Chilling Temperatures. Physiol Plant. 82. (4): 551–558. doi: 10.1111/j.1399-3054.1991.tb02946.x

Wang Z. H., Zong Z. Q., Li S. X., Chen B. M. (2002). Nitrate Accumulation in Vegetables and Its Residual in Vegetable Fields. Environ Sci. 23: 79–83.

Zhang E., Lin L., Liu J., Li Y., Jiang W., Tang Y. (2017). The Effects of Organic Fertilizer and Inorganic Fertilizer on Yield and Quality of Lettuce. Advances in Engineering Research 129: 907-910

Zhao L., Wang Y. (2017). Nitrate Assay for Plant Tissues. Bio-prot 7(2): e2029. doi: 10.21769/BioProtoc.2029.

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