Thesis of Doctoral (Ph.D.) Dissertation

EFFECT OF SOME NATURAL EXTRACTS ON DIFFERENT QUALITY PARAMETERS OF LETTUCE (*Lactuca sativa* L.) VARIETIES

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2022

1. BACKGROUND AND OBJECTIVES OF THE DOCTORAL DISSERTATION

Lactuca sativa L. is a leafy annual vegetable which is one of the most popular salad vegetables around the world. The plant is the major consumed vegetable in the human diet (Kim et al., 2016). Lettuce is commonly consumed raw due to its high vitamin (B_9, C and E), polyphenol, flavonoids, carotenoids, and mineral content (Mou, 2009, 2012). However, the nutritious value of lettuce is not appreciated since the plant is rich in water (around 95%) but low in calories (Kim et al., 2016). As a result, researchers are now interested in research studies about vitamin fortification and nutritional value in plants. Thus, numerous technological advancements have been proposed in recent decades to increase agricultural production system sustainability by drastically lowering synthetic agrochemicals such as pesticides and fertilizers.

Plant biostimulants are natural and environmentally friendly innovation that improves flowering, plant growth, fruit development, crop yield, and nutrient utilization efficiency, as well as resistance to a wide range of abiotic stresses (Rouphael and Colla, 2020). These studies are focusing more on the agronomical factors as use of plant biostimulants influencing yield and quality of different lettuce cultivars.

As a result, in order to reduce the environmental and human health impact of chemical fertilizers, biostimulants can be used as a supplementary fertilizer to improve plant quality and reduce the amount of chemical fertilizers required in plant production while also improving the plant's ability to absorb and translocate nutrients (Pascale et al., 2017; Zodape et al., 2011). To do this, three plant extracts, Moringa (*Moringa oleifera* Lam) leaf extract (MLE), Willow (*Salix babylonica* L.) bark extract (W) which are home-made extracts, and a manufactured extract Bistep (B), as well as the interaction of Willow and Bistep (W+B), are administered to three lettuce cultivars, "May King," "Kobak," and "Great Lakes." Biostimulants, depending on their content and composition, can be administered to plants at low quantities to the soil or the leaves (Kunicki et al., 2010).

Moringa leaf extract (MLE) is a recently discovered plant extract that is applied to plants as an environmentally friendly and safe biostimulant due to its high concentration of useful bioactive components, macro and micronutrients, minerals, plant hormones, essential amino acids, and vitamins (Rady et al., 2013; Sohaimy et al., 2015; Yaseen and Takácsné-Hájos, 2020).

Because plants are the primary source of vitamins, minerals, and other nutrients for the human body. Scientists are constantly searching for the best and safest materials to improve the quality parameters of plants, particularly vegetables. Commercially, quality parameters are increasingly important in global trade and marketable purchasing. Quality parameters are classified into two categories: outer (physical or morphological) and inner (nutritional value).

The main objectives of the research are:

- To investigate the effects of plant biostimulants Moringa leaf extract (MLE), Willow bark extract (W), Bistep (B), as well as their interaction Willow+Bistep (W+B) on morphological and bioactive compounds in three lettuce cultivars.
- To investigate the impact of Moringa leaf extract (MLE) on the nitrate content of lettuce leaves, resulted by reduce light intensity mostly in autumn season.
- Willow bark extract (W) is known as a biofungicide; however, its influence on physical quality parameters in lettuce has not yet been demonstrated. As a result, we wanted to test the effect on various lettuce varieties.
- The Bistep (B), which contains humic acid but little information on how to combine it with other biostimulants such as (Willow). We wanted to demonstrate the effect of combination Bistep (B) and Willow bark extract (W) on nutrient uptake and bioactive compounds in lettuce.
- We also wanted to investigate the interaction of genetic and climate factors on the evaluation of physical parameters, bioactive compounds, and mineral contents for successful lettuce production.

2. MATERIALS AND METHODS

2.1. Spring experiment

2.1.1. Plant materials and experimental design

Lettuce seeds were sown under the optimum weather condition in plastic trays under glasshouse in February 2019-2021 at University of Debrecen, Farm and Regional Research Institute, Botanical and Exhibition Garden, Hungary. Three different cultivars, "May King," "Kobak," and "Great Lakes," were chosen as plant materials for this experiment to be transplanted and grown in a plastic house. One month after the seed sowing, the seedlings with 4 true leaves were transplanted under unheated plastic house directly to the calcareous lowland chernozem soil which some chemical properties of the soil are shown in Table 1. The experiment was laid out based on factorial Randomized Complete Block Design (RCBD) with 3 replication and 20 seedlings per replication for each variety.

Table 1. Soil chemical properties at the experiment site in **spring** under plastic house

Soil chemical content	Years		
	2019	2020	2021
Soil pH (KCl)	7.35	7.28	7.56
Organic carbon (humus content) ($m\ m^{-1}$) %	2.88	2.40	1.72
P_2O_5 ($mg\ kg^{-1}$)	3137	1483	1317
K_2O ($mg\ kg^{-1}$)	459	374.02	291
Mg ($mg\ kg^{-1}$)	584	378.67	240
Na ($mg\ kg^{-1}$)	69.7	78.64	60.6
S ($mg\ kg^{-1}$)	98.7	56.70	42.1
$CaCO_3$ ($m\ m^{-1}$) %	4.20	3.21	0.72
NO_3^- ($mg\ kg^{-1}$)	130	53.97	244
Zn ($mg\ kg^{-1}$)	35.85	20.60	11.0
Cu ($mg\ kg^{-1}$)	9.54	6.88	4.39
Mn ($mg\ kg^{-1}$)	35.0	75.49	90.6
Total water-soluble salts ($m\ m^{-1}$) %	0.10	0.05	0.003
Soil plasticity (K_A)	42.00	37.72	33.00

Source: Agricultural Laboratory Centre, University of Debrecen, 2019-2021

2.1.2 Climate condition of the spring experiment

The temperature, light and relative humidity was lower in the beginning of transplanting the seedlings in March, then it was gradually increased. However, some large variations were recorded during the growing months of the lettuce growing season (Table 2).

Table 2. The average weather condition and lettuce growing practices under plastic house of the **spring** experiments (Debrecen, 2019-2021)

Years	Months		
	March	April	May
	Temperature (°C) MEAN		
2019	8.9	11.5	13.6
2020	7.0	13.3	14.9
2021	10.6	14.3	20.3
	Relative Humidity (RH%) MEAN		
2019	62.1	63.5	77.8
2020	57.4	49.7	63.9
2021	78.2	56.2	39.2
	Light emission (W/m^2) MEAN		
2019	149.8	141.9	141.9
2020	136.0	185.8	172.0
2021	173.3	194.6	244.4
	Transplanting		Harvesting
2019	25^{th}	-	16^{th}
2020	18^{th}	-	12^{th}
2021	20^{th}	-	11^{th}

Source: Agricultural Laboratory Centre, University of Debrecen

2.2. Autumn experiment

2.2.1. Plant materials and experimental design

For the autumn experiment in September 2019-2021, seeds from the same plant varieties "May King," "Kobak," and "Great Lakes" were sown under glasshouse environmental conditions. After the seedlings had four true leaves, they were transplanted directly into the glasshouse soil where the soil parameters listed in Table 3. The treatments [control and 6% Moringa leaf extract (MLE)] were applied once a week until harvest.

2.2.2. Climate condition of the autumn experiment

We regulate the glasshouse temperature using priva smart climate control computer software at 25 °C for seed germination until the seeds germinated, then the trays were transplanted to a location with a lower temperature than the growth chamber (15 to 20 °C). When the seedlings had 3 to 4 true leaves, they were transplanted to the glasshouse's direct soil in early November, where the temperature was similar (15 to 20 °C) and the relative humidity was above 50% (Table 4).

Table 3. Soil chemical properties at the experimental site in **autumn** seasons under glasshouse condition (Debrecen, 2019-2021)

Soil chemical content	Years		
	2019	2020	2021
Soil pH (KCl)	7.40	7.18	5.27
Organic carbon (humus content) (m m^{-1}) %	1.70	3.04	2.66
P_2O_5 (mg kg^{-1})	236	314	788
K_2O (mg kg^{-1})	177	381	499
Mg (mg kg^{-1})	239	192	388
Na (mg kg^{-1})	42	155	314
S (mg kg^{-1})	50.40	11.70	262
$CaCO_3$ (m m^{-1}) %	3.03	1.62	<0.100
NO_3^- (mg kg^{-1})	70.60	89.9	14.70
Zn (mg kg^{-1})	1.90	3.27	9.52
Cu (mg kg^{-1})	1.40	2.57	3.61
Mn (mg kg^{-1})	22	11.40	60.70
Total water-soluble salts (m m^{-1}) %	0.11	0.04	0.04
Soil plasticity (K_A)	31	38	40

Source: Agricultural Laboratory Centre, University of Debrecen

Table 4. The average weather condition during the lettuce vegetation period under glasshouse of the **autumn** experiments (Debrecen, 2019-2021)

Years	Months		
	October	November	December
	Temperature (°C) MEAN		
2019	17.7	17.8	17.7
2020	21.1	18.9	16.5
2021	12.2	15.3	15.5
	Relative Humidity (RH%) MEAN		
2019	63.1	65.5	55.9
2020	54.3	53.8	63.9
2021	84.2	68.8	65.7
	Light emission (W/m^2) MEAN		
2019	133.54	59.97	52.57
2020	87.68	58.24	40.84
2021	119.78	74.68	56.83
	Transplanting		**Harvesting**
2019	17th	-	21st
2020	27th	-	26th
2021	21st	-	23rd

2.3. Biostimulant treatments

2.3.1. Spring plant treatments

These experiments were conducted on a continuous basis for three years, from spring 2019 to spring 2021, at the Agrar Campus of the University of Debrecen in Hungary. Three lettuce cultivars ("May King," "Kobak," and "Great Lakes") were organised in a full randomized block design with three repetitions, with 20 plants chosen for each replication to be treated.

The following treatments were applied once every two weeks for four weeks throughout the growth season, beginning the first week after transplanting the seedlings. The chemical contents of the applied biostimulants are shown in Table 5.

Table 5. Some chemical contents of the plant biostimulants used in the experiments

Parameters	Bistep	Willow extract	Moringa leaf extract (MLE)	
			Fresh (Autumn)	Dry (Spring)
pH	7.40	7.20	5.05	7.49
Nitrate (NO_3^-) (mg L^{-1})	0.02	< 0.20	0.18	1.17
Phosphorus (P_2O_5) (mg L^{-1})	0.03	78.80	100	51.40
Potassium oxide (K_2O) (mg L^{-1})	0.30	23.50	94.40	37.70
Magnesium (Mg) (mg L^{-1})	0.02	6.02	86.80	10.50
Manganese (Mn) (mg L^{-1})	0.007	0.38	0.94	0.99
Zinc (Zn) (mg L^{-1})	0.008	0.07	0.48	1.63
Molybdenum (Mo) (mg L^{-1})	0.09	-	-	-
Organic material (%)	25.0	-	-	-
Iron (Fe) (mg L^{-1})	0.01	-	2.24	2.10
Sodium (Na) (mg L^{-1})	-	14.7	11.40	50.10
Boron (B) (mg L^{-1})	0.0002	-	0.58	0.25
Sulphur (S) (mg L^{-1})	-	5.21	57.00	17.10
Calcium (Ca) (mg L^{-1})	-	-	326	154
Copper (Cu) (mg L^{-1})	-	0.16	0.32	0.51
Total number of germs (number cm^{-3})	$0.8\ 10^7$	-	-	-
Micro fungus (number cm^{-3})	1.0×10^2	-	-	-

- **Control (untreated plants)**: the plants were sprayed with distilled water only.
- **Willow treatment (W)**: the plants were irrigated with Willow bark extract with the concentration of 3% with the amount of 50-70 mL $plant^{-1}$
- **Bistep treatment (B)**: Lettuce cultivars were foliar sprayed after mixing with 2 drops of detergent with the concentration of 0.05% with the amount of 15-20 mL $plant^{-1}$.

- **The combination of Willow bark extract (3%) and Bistep (0.05%) (W+B)**: the amount of 50-70 mL plant^{-1} (Willow bark extract 3%) + 15-20 mL plant^{-1} of 0.05% Bistep.
- **Moringa leaf extract (MLE)**: The plants were foliar sprayed with moringa leaf extract (6 % MLE) prepared in the leaf powder from moringa leaves stored in the end of August of the previous year from trees grown by ourselves, in the amount of 15-20 mL plant^{-1}.

Note: Because the moringa trees were not fully developed in 2019 to collect the leaves and prepare the extract for the treatment, the plants were only treated with Willow and Bistep biostimulants in the spring 2019 trial.

2.3.2. Autumn plant treatments

For the autumn experiment from 2019 to 2021, we only applied moringa leaf extract (6% MLE), which we prepared from the fresh moringa leaves grown by ourselves. So that, similar to the spring experiment, three lettuce cultivars ("May King," "Kobak," and "Great Lakes") were organised in a full randomized complete block design (RCBD) with three repetitions, 20 plants chosen for each replication to be treated. However, the foliar application was carried out every week instead of two weeks as follows:

- **Control (untreated plants)**: the plants were sprayed with distilled water.
- **Moringa leaf extract (MLE)**: the plants were foliar sprayed with the moringa leaf extract (6% MLE) prepared in the fresh leaves with the amount of 15-20 mL plant^{-1}.

2.4. Morphological and sensory evaluations

The physical parameters were measured at the day of harvest. The measurements were carried out for the following criteria:

- Head weight: (g head^{-1}).
- Root weight: (g root^{-1}).
- Head diameter: (cm)
- Internal stem size: (cm)
- Number of leaves: (piece head^{-1})

Whereas, the sensory evaluation was caried out based on the following criteria:

- Head structure: (1 – loose …. 10 – firm)
- Closing of heads: (1 – very opened…. 10 – very closed or compacted).

2.5. Laboratory measurements

Macro and micro-nutrient analysis

Total dry matrix content was determined by drying the samples at 105 °C until mass constancy was reached (min. 4 h). Microelements (Ca, K, Mg, Na, S, and P) were determined using the ICP-OES (iCAP 7400, Thermo Scientific) technique. From the properly prepared sample, 0.5 g was measured in a high-pressure Teflon bomb. 5 ml of distilled cc. HNO_3 and 3 ml of 30% H_2O_2 were added. It has been sealed and digested in Ethos Plus Microwave Digestion System (Milestone) applying a method (Application Note 076) of the manufacturer [3 min 85°C, 9 min 145°C, 4 min 200°C, 14 min 200 °C]. After cooling the Teflon bombs, the digested samples were diluted to 50 ml, homogenized and filtered (MN 640 W, Millipore) before element analysis. This was based on the method by Krüger et al. (2014).

Determination of total polyphenol content

Total polyphenols in mg Gallic acid equivalent (GAE) 100 g^{-1} fresh product were determined by Folin-Ciocalteu spectrophotometric method with gallic acid reference material based on the method by Meda et al., (2005).

Determination of Vitamin C content

The sample was homogenized, and 10.0 to 20.0 g was weighed. Ascorbic acid was extracted from the weighed sample with oxalic acid solution and subsequently titrated with the 2,6 dichlorophenol-indophenol to salmon pink colour. Ascorbic acid content was calculated from the added volume (Ciancaglini et al., 2001).

Determination of nitrate

Frozen samples were homogenized, and 40 g was measured. 35 g extraction buffer ([5% (v/v) HCl and 7.5% (v/v) ammonium, pH 9.6-9.7] and 325 g distilled water were added to the sample. The mixture was homogenized for 1 min and filtered through filter paper, and the filtrate was freshly analysed for the nitrate content. The general principle of the nitrate (NO_3^-) determination is using multichannel Continuous Flow Analyzer (CFA, FIASTAR 5000, Foss, Germany) on 540 nm after the reduction in copper coated cadmium column (NO_3^- + 2^{e-} ® NO_2^-) to form diazo compound with colour-developing Griess reagent based on the method by (Kmecl et al., 2007).

2.6. Determination of SPAD and NDVI

A portable chlorophyll meter SPAD-502 (Konica Minolta, Japan) was used to measure the chlorophyll content and normalized difference vegetation index (NDVI) in lettuce cultivars using the technique developed by (Madeira et al., 2003). Chlorophyll content can be evaluated by non-destructive method as SPAD instrument instead of destructive method (Ali et al., 2021). Ten fully mature lettuce plants from each cultivar were randomly measured in three leaves each (middle leaves in three directions), and the average was used as a single SPAD vale for statistical analysis. The trimble GreenSeeker handheld crop sensor Model HSC-100 (Trimble Navigation Unlimited, Sunnyvale, CA), on the other hand, was utilized to measure the NDVI value at the same vegetable growth stage by scanning twenty plants in each variety and treatment at a height of about one meter above the ground. The data was then recorded for each plant for examination and review.

2.7. Statistical analysis

The data from the three-year study's spring experiment (2019-2021) were subjected to statistical analysis using one-way analysis of variance (ANOVA) in SPSS (SPSS Inc., Chicago, IL, USA) computer program, version 25.0 (IBM Corp. Released, 2017). At a significance threshold of $p \geq 0.05$, the Duncan[a,b] multiple range test was performed to validate the differences between the compared averages. To assess if there were significant differences between the two groups (In the autumn experiments), the means of five samples were subjected to the independent sample *t-test* at a significance threshold of $p \geq 0.01$ for the glasshouse experiments.

3. RESULTS

Morphological parameters

Effect of plant biostimulant on vegetative parameters of lettuce

The most important marketable parameters, which are among the first consumer attractions to purchase fresh lettuce, were evaluated. Our three-year mean results show that there are no significant changes in lettuce head weight in plants treated with plant biostimulants. Plant biostimulants such as Willow bark extract (W), Bistep (B), and Willow bark extract + Bistep (W+B) did, however, slightly increase the head weight of several lettuce cultivars when compared to control plants. In contrast, a slight reduction in head weight was observed in lettuce treated with moringa leaf extract (MLE) (Figure 1).

Significant differences were mostly seen between the growing years, with some biostimulants influencing better in cooler environments than in warmer. The best spring season result was obtained among the growing years (2019-2021) depends on the weather condition, when the temperature was cooler throughout the growth months of March, April, and May (Table 2).

On the other hand, the plant biostimulants had no significant influence on the head diameter and root weight (Figure 1).

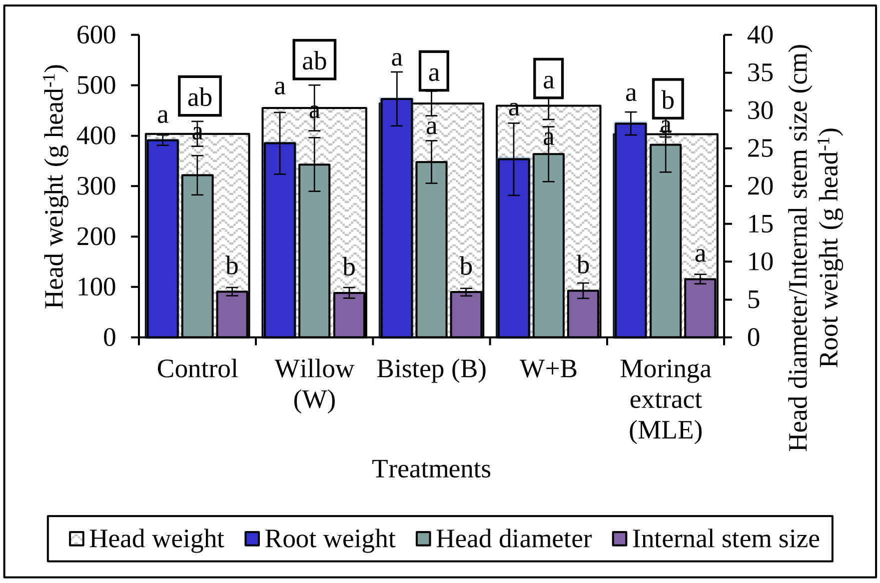

Figure 1. Effect of plant biostimulants on some measured vegetative parameters of lettuce in the mean of varieties in **spring** of 3 years, (Debrecen, 2019-2021)

However, the internal stem size of the treated plants was enhanced by 6 % MLE, which might be because moringa leaf extract can influence better in warmer climate conditions than other plant biostimulants. This means that those biostimulants could improve the heat tolerance in the lettuce cultivars under specific weather conditions (Figure 1).

We repeated similar experiments in the autumn using only 6% MLE to compare the effect of plant biostimulants on the measured morphological parameters. In general, the physical parameters measured in autumn were much lower than in spring. The mean results from autumn experiments show that a plant biostimulant of 6% MLE has a similar effect on the measured quality parameters (Figure 2). Some lettuce morphological parameters improved, including head weight, head diameter, internal stem size, and root weight. There were, however, no discernible differences. The only significant differences were found between growing years.

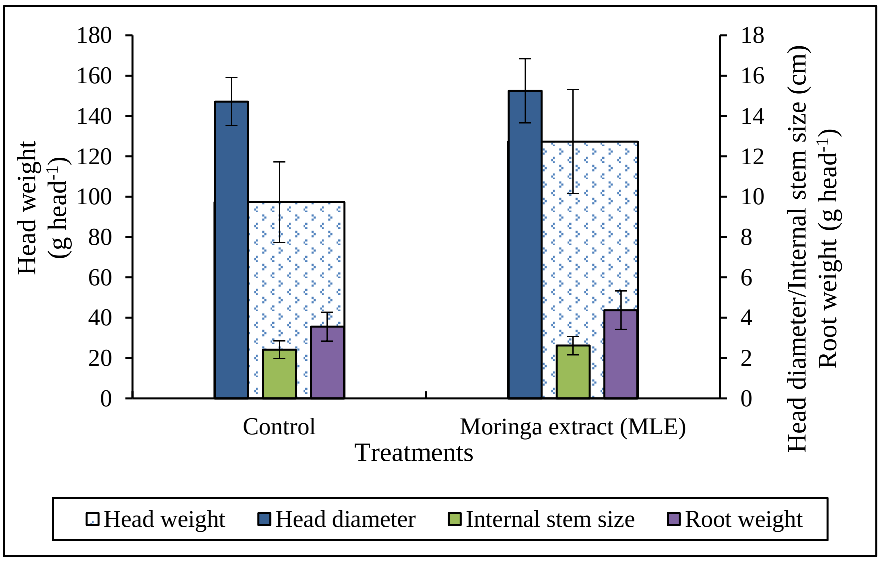

Figure 2. Effect of plant biostimulants on some measured vegetative parameters of lettuce in the mean of varieties in **autumn** of 3 years, (Debrecen, 2019- 2021)

Effect of plant biostimulant on some sensory vegetative parameters of lettuce

The scaled method was used to measure head structure and head closure, as detailed in chapter of Materials and Methods. These features are essential for consumers purchasing products in markets. This physical attribute improves the appearance of the lettuce and entices customers purchases. Our mean results for spring seasons in Figure 3 demonstrate that all of the data indicate that the treated plants could have a better head structure, but only the treated plants with Willow bark extract (W) showed a significant improvement.

Furthermore, the head closing or the compacting of the heads or head opening is also measured because they are also important for the consumer buying and retail packaging. Similar to the head structure, the head closing was positively influenced by plant biostimulant treatments for all three years. However, among the treatments, the significant results were performed for the lettuce varieties treated with Bistep, W+B and MLE, whereas no significant difference was seen in Willow (W) treatment (Figure 3). The growing years also had an effect on the two above-mentioned scaled parameters, where the biostimulants could have a significant influence on the head structure in 2020, whereas, with the exception of Willow bark extract, other biostimulants could significantly improve head closing. We also noticed a wide range of reactions to plant biostimulants during our three-year study. Leaf number, on the other hand, was mostly impacted by genetic variations rather than plant biostimulants. There was a minor variation in leaf number, but it was not statistically significant. For example, the mean of growing years shows that plants treated with 6% MLE had the highest leaf number (40.44 leaves plant^{-1}), whereas MLE could significantly increase the number of leaves in 2020 (46.6 leaves plant^{-1}), and the combination of Willow + Bistep (W+B) produced the highest number of leaves in 2021 (38.5 leaves plant^{-1}).

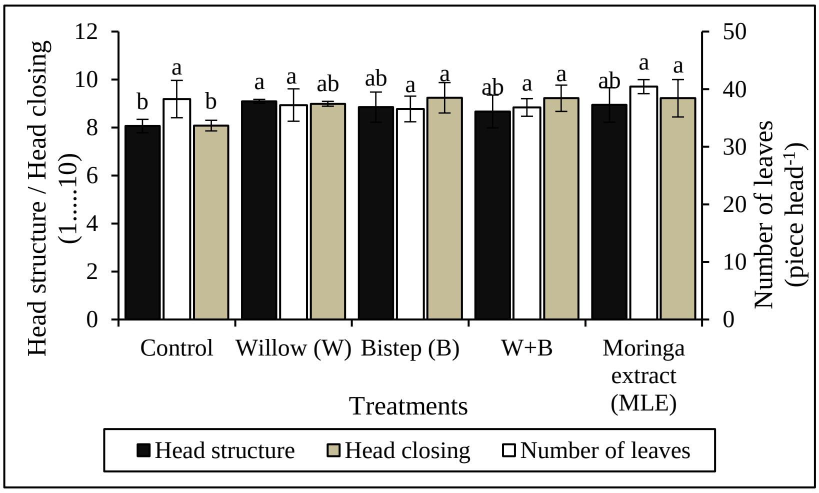

Figure 3. Effect of plant biostimulants on some scaled vegetative parameters of lettuce in the mean of varieties in **spring** time, (Debrecen, 2019- 2021)

The mean results of the autumn growing season, on the other hand, show no significant improvement in scaled morphological parameters and leaf number (Figure 4). The head structure and number of leaves of lettuce cultivars treated with moringa leaf extract (6% MLE) show a slight improvement, while head closure is similar between treated and untreated plants.

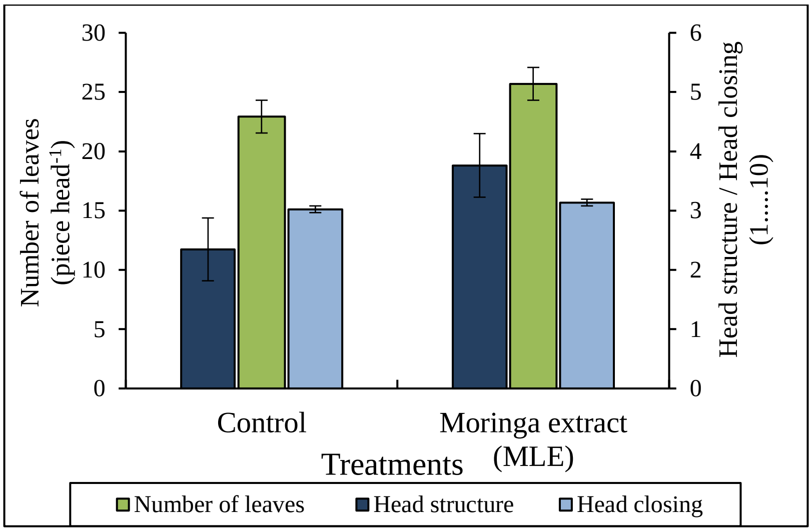

Figure 4. Effect of plant biostimulants on some scaled vegetative parameters of lettuce in the mean of varieties in **autumn** season, (Debrecen, 2019- 2021)

Effect plant varieties on some measured vegetative parameters

Plant varieties are distinguished primarily by their physical characteristics. We evaluated three different lettuce cultivars with different genotypes in our research studies: Butterhead Lettuce "May King," Batavia type "Kobak," and Iceberg type "Great Lakes" cultivars because each one has different vegetative characteristics. Plant phenotypes can be influenced by both genetic and environmental factors, as well as their interactions (Fasahat et al., 2015; Mou, 2009).

Figure 5 indicates that throughout the growth seasons, the "Great Lakes" variety produced significantly higher head weight (g head^{-1}), although no significant variations were identified between the "May King" and "Kobak" cultivars. On the other hand, there were no significant differences in head diameter amongst the three lettuce cultivars; the only significant differences were between the growing years of 2019 and 2020, with the "May King" variety having the biggest head diameter in 2020 at 31.25 cm head^{-1}.

There was also a significant difference in internal stem size (cm head^{-1}) among cultivars, which was mostly related to the number of leaves. Plants with more leaves, such as "Kobak" have a longer internal stem size than other cultivars since the leaves in lettuce vegetable are closely tied to the stem. In contrast, no substantial variation in root weight was identified across the examined cultivars; the only changes were attributed to growing years. For example, in 2020, the most root weight was measured in the "May King" variety, while in 2021, it was in the "May King" and "Kobak" cultivars, which might be due to lower relative humidity in the soil.

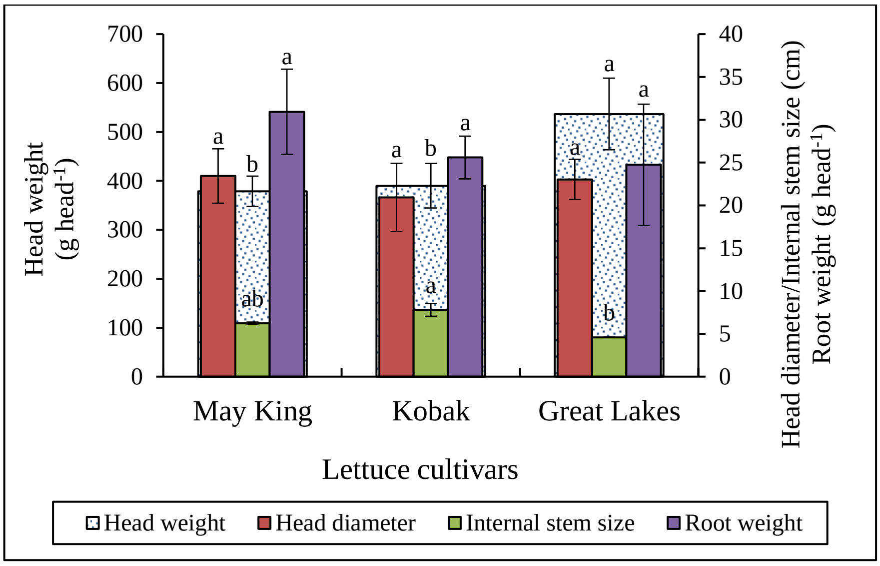

Figure 5. Effect of lettuce genotype on some measured vegetative parameters of lettuce in the mean of treatments in **spring** season, (Debrecen, 2019-2021)

In contrast, the means of growing years show that lettuce cultivars grown in autumn seasons had no statistically significant differences in the measured morphological parameters (Figure 6). However, changes were mostly observed over time based on environmental factors, particularly light intensity. For example, the significantly largest head weight was measured in the growing year of 2021, mostly in the "Great Lakes" variety, which could be attributed to higher light emission in the late growing months of November and December (Table 4). Similar results were observed for the other measured physical parameters.

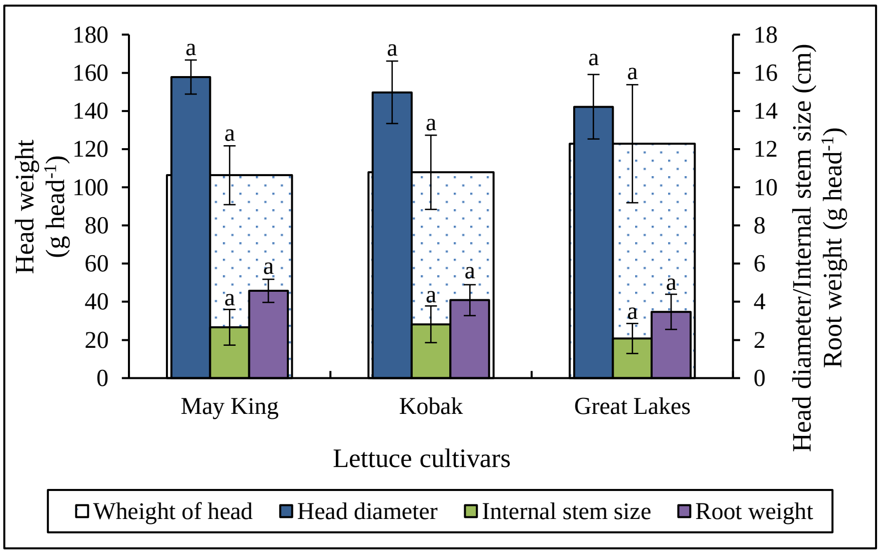

Figure 6. Effect of lettuce genotype on some measured vegetative parameters of lettuce in the mean of treatments in **autumn** season, (Debrecen, 2019- 2021)

Effect of lettuce varieties on some scaled vegetative parameters of lettuce

The phenotypic appearance in lettuce is determined by environmental (E) and genetic (G) factors, as well as their interactions (Burns, et al., 2011). The exterior forms or head development of lettuce in the same cultivars are clearly distinguishable. Some produce well-formed heads, known as Crisphead as in "Great Lakes", while others produce unformed or mostly divided leaves, known as Butterheads as "May Kings" or Batavia types as "Kobak" varieties. In the spring seasons, our mean result shows that the genotypes had the same result for head structure and head closure (Figure 7). However, there were significant differences between growing years, with the "May Kings" and "Kobak" varieties producing significantly better head structure and head closing compared to "Great Lakes," while the "Kobak" variety produced significantly poorer head structure and head closing in 2021.

The number of leaves, on the other hand, was perhaps the most strongly influenced morphological parameter by genotype differences. The "Kobak" lettuce variety produces a significantly higher number of leaves, followed by "May Kings," and the "Great Lakes" lettuce variety had the lowest number of leaves.

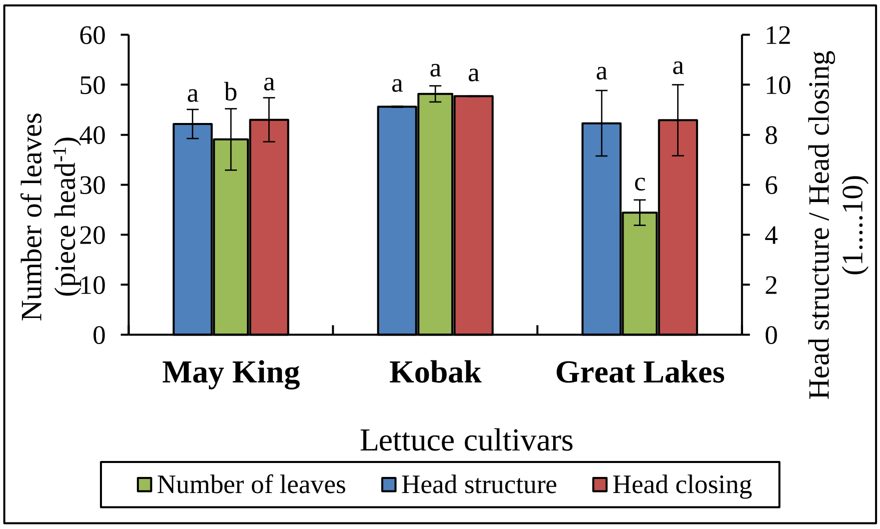

Figure 7. Effect of lettuce genotype on some scaled vegetative parameters of lettuce in the mean of treatments in **spring** season, (Debrecen, 2019- 2021)

Lettuce cultivars grown in autumn season had a similar result on the head structure, head closing and number of leaves as it was grown in spring season. The mean results show the only significant variation in the number of leaves, whereas no significant differences were recorded for the head structure and head closing (Figure 8). However, the significant better head structure and head closing was seen in the growing years of 2019 and 2020 where the glasshouse temperature was lower than in 2021.

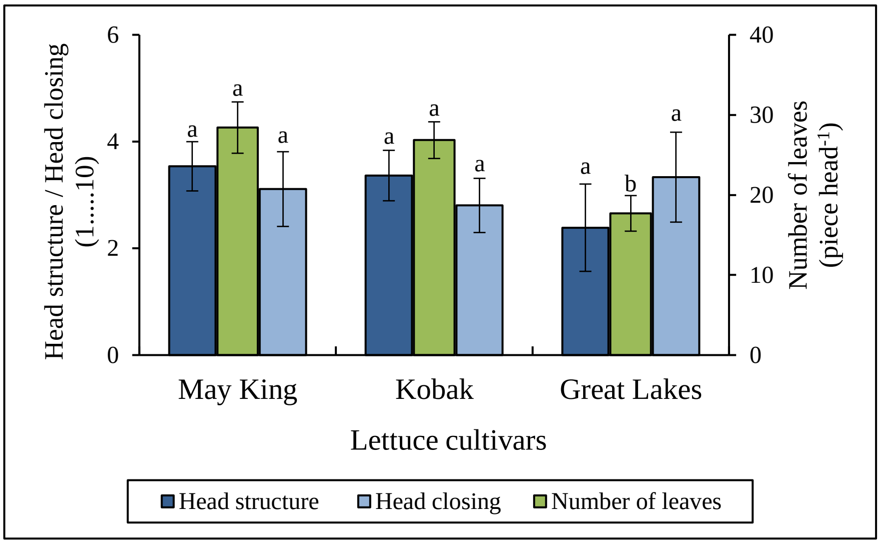

Figure 8. Effect of plant biostimulants on some scaled vegetative parameters of lettuce in the mean of varieties in **autumn** season, (Debrecen, 2019-2021)

Mineral element content

The interaction effect of plant biostimulants and growing years on macronutrient content in three lettuce cultivars

Lettuce seems to be an essential source of the human diet. However, when compared to other green vegetables such as spinach, the plant is nutritionally underestimated (Kim, et al., 2016). It is essential to increase the mineral content of the plant using environmentally friendly biostimulants without raising the nitrate content of the leaves. As shown in Table 6, foliar spray and irrigation treatment of the applied plant biostimulants considerably boosted various macronutrients in lettuce cultivars.

Willow bark extract (W) and the combination of Willow+ Bistep (W+B) were the most efficient biostimulants in enhancing nutritional content in lettuce cultivars when compared to untreated plants and Bistep (B).

A significant improvement is shown in the combination of W+B significantly enhanced calcium (Ca) content in "May King" and "Kobak" over a period of years. Plants treated with Willow bark extract and W+ B, on the other hand, had considerably higher levels of magnesium (Mg), sodium (Na), and phosphorus (P). In contrast, no significant effects were observed for the potassium concentration in lettuce genotypes for Willow (W) and the combination of W+B treatments. Despite the fact that the Bistep biostimulant reduced K accumulation in all three cultivars. Willow bark extract significantly improved the macronutrients Ca, K, and P in colder seasons (2019) compared to warmer seasons (2020 and 2021). During the seasons, however, higher levels of Na and Mg were discovered in lettuce treated with Willow extracts. In addition to this, Willow bark extract+ Bistep (W+B) significantly raised Ca, Mg, P, and S throughout the grown years, while Willow bark extract (W) also significantly enhanced Na in lettuce cultivars. This might be because Willow bark extract (W) was applied to the plant via irrigation, allowing the roots to absorb more nutrients from the soil, and the extract itself is high in minerals (Table 6).

Table 6. Macronutrient content in lettuce in related to plant biostimulants

Treatments	Cultivars			Years			Mean of years
	May King	Kobak	Great Lakes	2019	2020	2021	
Potassium (mg kg^{-1} DW)							
Control	1352.33^{a}	1390.00^{a}	1485.15^{a}	1124.00^{c}	1772.47^{a}	1331.00^{a}	1409.16^{a}
Bistep	1106.85^{b}	1006.34^{b}	1137.85^{b}	997.35^{d}	1318.30^{d}	935.38^{b}	1083.70^{d}
Willow	1164.23^{ab}	1374.52^{a}	1302.46^{b}	1303.37^{a}	1579.45^{b}	958.38^{b}	1280.41^{b}
W+B	1186.32^{ab}	1232.85^{a}	1176.84^{b}	1230.85^{b}	1421.66^{c}	943.50^{b}	1198.67^{c}
Calcium (mg kg^{-1} DW)							
Control	419.74^{b}	422.00^{bc}	491.75^{ab}	500.33^{b}	492.16^{b}	314.00^{d}	444.48^{b}
Bistep	411.77^{b}	399.59^{c}	476.85^{ab}	468.90^{c}	441.33^{c}	378.00^{c}	429.40^{b}
Willow	410.74^{b}	458.74^{b}	463.73^{b}	517.00^{a}	400.46^{d}	415.75^{b}	444.40^{b}
W+B	521.59^{a}	540.10^{a}	505.76^{a}	506.83^{ab}	573.53^{a}	487.08^{a}	522.48^{a}
Phosphorus (mg kg^{-1} DW)							
Control	269.66^{b}	281.33^{ab}	262.03^{ab}	240.17^{ab}	292.00^{bc}	280.86^{ab}	271.01^{b}
Bistep	261.29^{b}	258.68^{b}	270.52^{ab}	227.64^{b}	300.00^{b}	262.85^{b}	263.51^{b}
Willow	259.66^{b}	278.50^{ab}	260.02^{b}	255.51^{a}	275.33^{c}	267.34^{b}	266.06^{b}
W+B	298.33^{a}	299.00^{a}	287.67^{a}	246.01^{ab}	338.33^{a}	300.66^{a}	295.01^{a}
Magnesium (mg kg^{-1} DW)							
Control	231.52^{b}	228.81^{c}	251.00^{b}	279.77^{b}	246.66^{c}	184.88^{d}	237.12^{d}
Bistep	252.66^{b}	256.69^{b}	270.80^{b}	268.33^{b}	273.53^{b}	238.30^{c}	260.05^{c}
Willow	282.13^{a}	307.50^{a}	310.06^{a}	306.33^{a}	322.86^{a}	270.51^{b}	299.90^{b}
W+B	301.97^{a}	315.53^{a}	307.50^{a}	307.66^{a}	283.68^{b}	333.66^{a}	308.33^{a}
Sodium (mg kg^{-1} DW)							
Control	72.94^{c}	78.45^{c}	91.33^{c}	106.82^{c}	80.36^{d}	55.54^{c}	80.91^{d}
Bistep	100.75^{b}	104.55^{b}	116.50^{b}	76.96^{d}	140.36^{c}	104.48^{ab}	107.26^{c}
Willow	123.23^{ab}	152.67^{a}	141.82^{a}	132.72^{a}	176.77^{a}	108.23^{a}	139.24^{a}
W+B	131.67^{a}	125.44^{b}	127.38^{b}	121.66^{b}	163.22^{b}	99.61^{b}	128.16^{b}
Sulphur (mg kg^{-1} DW)							
Control	94.75^{b}	113.93^{a}	105.19^{a}	109.17^{c}	118.07^{b}	86.64^{a}	104.63^{b}
Bistep	89.17^{b}	88.67^{b}	106.76^{a}	92.19^{d}	117.16^{b}	75.25^{b}	94.87^{d}
Willow	88.00^{b}	105.94^{ab}	109.65^{a}	127.55^{a}	104.62^{c}	71.44^{b}	101.20^{c}
W+B	118.28^{a}	112.32^{a}	108.98^{a}	115.85^{b}	138.22^{a}	85.51^{a}	113.19^{a}

Means within the same column followed by the same letter(s) are not significantly different ($P \leq 0.05$) according to Duncan^{a,b} test Multiple Range Test

The results from mean of years show that the combined treatment of W+B significantly increased most of the measured macronutrients, with calcium, magnesium, phosphorus, and sulphur being the key macronutrients. The treatment of W+B, on the other hand, dramatically lowered potassium levels. Willow bark extract, on the other hand, considerably improved sodium levels which might be due to its high sodium content (14.7 mg L^{-1}).

The interaction effect of plant biostimulants and growing years on micronutrient content in three different lettuce cultivars

Many physiological activities in plant cells, including enzymatic processes, respiration, photosynthesis protein stability, and redox transition elements, are influenced by micronutrients (Salih, 2021). The results in the Table 7 demonstrate that similar to the macronutrient, the micronutrient was significantly influenced by the combination treatment of plant biostimulants and the growing years.

Table 7. Micro nutrient content in lettuce in related to plant biostimulants

Treatments	Cultivars			Years			Mean
	May King	Kobak	Great Lakes	2019	2020	2021	
Iron (mg kg^{-1} DW)							
Control	21.55^{b}	22.48^{b}	25.75^{b}	18.17^{ab}	37.93^{b}	13.68^{b}	23.25^{b}
Bistep	20.24^{b}	19.50^{b}	21.11^{b}	16.47^{b}	29.65^{c}	14.72^{b}	20.28^{c}
Willow	23.78^{ab}	23.46^{b}	23.90^{b}	18.52^{a}	37.55^{b}	15.07^{b}	23.71^{b}
W+B	34.07^{a}	37.39^{a}	37.31^{a}	18.14^{ab}	68.46^{a}	22.16^{a}	36.26^{a}
Zinc (mg kg^{-1} DW)							
Control	2.694^{b}	2.882^{a}	2.915^{a}	2.723^{ab}	2.843^{bc}	2.922^{a}	2.833^{ab}
Bistep	2.605^{b}	2.765^{a}	2.735^{a}	2.566^{b}	2.754^{c}	2.786^{b}	2.700^{c}
Willow	2.594^{b}	3.104^{a}	2.768^{a}	2.834^{a}	2.935^{ab}	2.696^{b}	2.822^{b}
W+B	2.840^{a}	2.928^{a}	2.957^{a}	2.720^{ab}	3.022^{a}	2.992^{a}	2.900^{a}
Manganese (mg kg^{-1} DW)							
Control	1.490^{bc}	1.753^{b}	1.929^{a}	1.735^{a}	1.582^{c}	1.854^{c}	1.724^{c}
Bistep	1.412^{c}	1.274^{c}	1.472^{b}	1.303^{c}	0.978^{d}	1.877^{bc}	1.385^{d}
Willow	1.652^{b}	1.720^{b}	1.973^{a}	1.653^{b}	1.732^{b}	1.960^{b}	1.783^{b}
W+B	2.105^{a}	2.510^{a}	2.073^{a}	1.751^{a}	2.561^{a}	2.377^{a}	2.230^{a}
Copper (mg kg^{-1} DW)							
Control	0.727^{a}	0.740^{ab}	0.903^{a}	0.706^{ab}	0.892^{b}	0.772^{a}	0.790^{b}
Bistep	0.730^{a}	0.669^{b}	0.978^{a}	0.704^{ab}	1.000^{a}	0.674^{b}	0.792^{b}
Willow	0.741^{a}	0.747^{ab}	0.974^{a}	0.736^{a}	1.027^{a}	0.698^{b}	0.821^{a}
W+B	0.719^{a}	0.787^{a}	0.945^{a}	0.684^{b}	1.021^{a}	0.746^{a}	0.817^{a}

Means within the same column followed by the same letter(s) are not significantly different ($P \leq 0.05$) according to Duncan[a,b] test Multiple Range Test

Iron content was clearly influenced by biostimulant treatments. Willow bark extract + Bistep (W+B) for example, recorded a significantly greater iron content compared to control. The only significant difference for zinc content was found in the "May King" lettuce variety treated with W+ B treatments at 2.84 mg kg^{-1} DW. Compared to the control plants, there was a significantly higher manganese content in the "May King" and "Kobak" treated with W+ B, manganese was also higher in the "Great Lakes" variety however, no significant difference was recorded. In contrast, foliar spray of Bistep significantly reduced the manganese content in the lettuce cultivars. However, in the mean of years, there was no significant difference in the copper content in the lettuce varieties treated with the plant biostimulants. A similar result was found on the broccoli treated with biostimulants under drought weather conditions on micro and macronutrient content by Kałużewicz et al. (2018).

In addition, the treatments, lettuce genotypes, and growing seasons all had an effect on iron content, which is one of the most important micronutrients in leafy vegetables. As shown in Table 7, W+B could greatly increase the iron content of all lettuce cultivars. Furthermore, among the lettuce cultivars, the "Great Lakes" variety had more iron than the "May King" and "Kobak" cultivars.

In the mean of cultivars, there was no substantial variation in zinc and copper content in lettuce types in 2019, although plant biostimulants could greatly enhance copper content in 2020. Willow+ Bistep (W+B) considerably increased manganese and zinc content in 2020 and 2021, which might be attributed to warmer weather and greater light in the late season of April and May before harvesting (Table 7). The results of the growing years demonstrate that Willow and W+B have the greatest influence on the micronutrient content of lettuce cultivars, whereas Bistep had the least impact on the micronutrient content.

Bioactive compounds

The effect of plant biostimulants on total polyphenol, vitamin C and nitrate content in lettuce leaves in the mean of cultivars

Phenolic chemicals are a form of secondary metabolite derived from plants that serve significant physiological activities throughout the plant's life cycle. Phenolics are produced under both favourable and suboptimal environments. Under abiotic stress circumstances, plants demonstrate enhanced synthesis of polyphenols such as phenolic acids and flavonoids, which aid the plant in coping with environmental restrictions (Sharma et al., 2019).

The results in Table 8 show that the impact of plant biostimulants on total polyphenols in lettuce cultivars varied depending on the growing years and treatments. The results reveal that plants treated with Willow bark extract + Bistep (W+B) considerably increased total polyphenols, although no significant difference was found for other plant biostimulants compared to the control.

Furthermore, the mean of treatments indicates a significantly higher total polyphenol in the year 2019. This might be due to the fact that the temperature in 2019 was a bit lower throughout the growing season than in 2020 and 2021.

Table 8. Effect of plant biostimulants on evaluation of bioactive compound in the mean of variety grown in **spring** season (Debrecen 2019-2021)

Treatments	**Bioactive compounds**											
	Total polyphenols (mg GAE 100g^{-1} FW)				**Vitamin C (mg 100 g^{-1} FW)**				**Nitrate (mg kg^{-1})**			
	2019	**2020**	**2021**	**Mean**	**2019**	**2020**	**2021**	**Mean**	**2019**	**2020**	**2021**	**Mean**
Control	55.85^{b}	57.48^{a}	60.15^{a}	57.82^{b}	4.87^{b}	4.68^{bc}	7.37^{b}	5.64^{c}	488.21^{b}	682.66^{b}	812.88^{b}	661.25^{b}
Willow	59.41^{b}	50.54^{b}	55.53^{ab}	55.16^{b}	5.29^{b}	5.27^{a}	8.11^{a}	6.23^{ab}	692.15^{a}	862.00^{a}	850.33^{a}	801.49^{a}
Bistep	55.60^{b}	57.44^{a}	52.73^{bc}	55.26^{b}	5.44^{b}	5.09^{ab}	6.94^{bc}	5.82^{bc}	527.81^{b}	604.00^{c}	815.88^{b}	649.23^{b}
W+B	80.64^{a}	52.46^{b}	57.09^{ab}	64.40^{a}	7.31^{a}	5.37^{a}	7.02^{bc}	6.57^{a}	536.50^{b}	659.00^{b}	786.55^{b}	660.68^{b}
MLE	-	60.88^{a}	51.73^{c}	56.31^{b}	-	4.55^{c}	6.71^{c}	5.63^{c}	-	680.32^{b}	810.11^{b}	745.21^{b}
Mean	62.87^{a}	55.76^{b}	55.45^{b}	-	5.72^{b}	4.99^{b}	7.23^{a}	-	561.17^{c}	697.59^{b}	815.15^{a}	-

Means within the same column followed by the same letter(s) are not significantly different at the probability level of (($P \leq 0.05$) according to Duncan [a,b] Multiple Range Test
FW: Fresh weight
W+B: Willow+Bistep

In 2019, the only significant difference was discovered for the combination of plant biostimulants of Willow bark extract + Bistep (W+B), whereas in 2020 a significantly reduction of total polyphenol was measured in the plants treated with Willow bark extract+ Bistep (W+B) and moringa leaf extract (MLE) plant biostimulants. This might be because these biostimulants can reduce heat stress in warmer weather conditions (Latif and Mohamed, 2016; Nayanakantha et al., 2019). The means show that the most influenced biostimulant on the total polyphenol improvement was Willow bark extract + Bistep (W+B). This might be because the combination of the treatments could provide all the necessary elements for the plant's physiological metabolisms.

Our result mean of years shows that in the mean of varieties all the plant biostimulants could improve the vitamin C in lettuce leaves. However, compared to the control or untreated lettuce cultivars Willow bark extract (W) and Willow bark extract+ Bistep (W+B) could significantly increase vitamin C, whereas no significant differences were recorded for the Bistep and MLE plant biostimulants. Total polyphenols and vitamin C had a negative connection, with an increase in total polyphenols lowering vitamin C levels in lettuce leaves.

In the year of 2019, only cultivars treated with Willow bark extract+ Bistep (W+B) had substantially higher vitamin C content at 7.31 mg 100 g^{-1} FW, but in 2020 plant biostimulants of Willow and Willow bark extract + Bistep (W+B) significantly enhanced vitamin C content in lettuce leaves by 11% and 13%, respectively. The maximum vitamin C content was found in plants treated with Willow bark extract (W) during the 2021 growing season, at 8.11 mg 100 g^{-1} FW. Our findings correspond with the findings by Schmidt et al. (2010) that the antioxidant content in Kale (*Brassica oleracea* var. *sabellica*) vegetable was most impacted and improved by climatic conditions, particularly cold temperatures from October to December.

According to our results, *Lactuca sativa* treated with Willow bark extract (W) significantly accumulates more nitrate content in their leaves throughout a three-year study. Among the plant biostimulants, Bistep (B) was the only biostimulant which could reduce nitrate level in spring growing season by around (-)13%.

In the mean of treatments, a significant greater nitrate level was measured in the growing year of 2021, while the light emission and temperature was higher than in 2020 and 2019. This might be because Willow bark extract (W) contains more nitrogen than the Bistep plant biostimulants at <0.20 mg L^{-1} in Willow, but only 0.02 mg L^{-1} in Bistep (Table 5), this will provide the plant to absorb and retain more nitrogen in its leaves. Drobek et al. (2019) have found a similar result on the corn treated with the combination of fertilizer (300 kg N ha^{-1} + 120 kg K ha^{-1}) and protein hydrolysate biostimulant of chicken feathers (7.2 L ha^{-1}) where a higher nitrogen level was provided to the plants.

Although lettuce grown in the autumn season has higher nitrate accumulation than lettuce grown in the spring season (Colla et al., 2018), vitamin C and total polyphenols may be unaffected by the growing season. In this case, we repeated the experiments in the autumn season and in different surroundings (under glasshouse condition) to see how the growing season and MLE treatment affected the bioactive compounds and nitrate levels in the lettuce varieties.
As it is shown in Table 9, the changes in total polyphenol, vitamin C, and nitrate content during the autumn season in relation to the plant biostimulant application of 6% MLE, lettuce genotypes, growing years, and their interactions.

The total polyphenol content in lettuce cultivars was less affected by the aforementioned factors, but the changes were most noticeable among growing years. The significant variation of the total polyphenols can be seen in the growing seasons of 2019 and 2020, whereas no changes were found in 2021. In the season of 2019, 6% MLE could significantly reduce the total polyphenol content, whereas in 2020 6% MLE could significantly improve the total polyphenol content. This could be because the temperature in 2019 fluctuated less and the plants were less stressed than in the 2020 season (Table 4). The interaction of plant biostimulants, lettuce cultivars, and growing years results in a slight increase in total polyphenols in plants treated with 6% MLE compared to untreated plants. The same was true for the vitamin C content. However, few differences were observed for the 2020 growing season. This is primarily due to the fact that the growing year of 2020 was significantly warmer than the other two years, 2019 and 2021. In general, the year 2020 had the highest total polyphenols and vitamin C content, while the year 2019 had the lowest. This could be due to environmental factors such as higher temperatures and lower light emission during the 2020 growing season.

Table 9. The interaction effect of plant varieties, treatments and growing years on total polyphenol, vitamin C and nitrate content in lettuce vegetable grown in **autumn** seasons from 2019-2021. (Debrecen, 2019- 2021)

Treatments	Varieties	Bioactive compounds											
		Total polyphenols (mg GAE $100g^{-1}$ FW)				Vitamin C (mg 100 g^{-1} FW)				Nitrate (mg kg^{-1})			
		2019	**2020**	**2021**	**Mean**	**2019**	**2020**	**2021**	**Mean**	**2019**	**2020**	**2021**	**Mean**
Interaction between MLE foliar application and plant variety													
May King	**Control**	37.65	51.63	51.40	46.89	5.85	6.03	9.72	7.20	784.00*	439.00*	1281.00*	834.66*
	MLE	35.59	65.83	48.40	49.94	4.81	9.21*	8.85	7.62	692.00	379.00	964.00	678.33
Kobak	**Control**	37.95*	65.4	48.90	50.75	5.83	8.87	8.99	7.89	950.00*	642.00*	1046.00*	879.33*
	MLE	35.61	70.88	47.60	51.36	4.91	9.92	8.59	7.80	772.00	517.00	876.00	721.66
Great Lakes	**Control**	41.73*	55.69	39.60	45.67	8.61	10.30*	6.19	8.36	1180.00*	702.00*	986.00*	956.00*
	MLE	37.26	73.03*	37.50	49.26	8.30	7.78	5.63	7.23	820.00	489.00	840.00	716.00
In the mean of cultivars													
Control		39.11*	58.93	46.63	48.22	6.76	7.28	8.30	7.44	971.55*	594.33*	1104.33*	890.00*
MLE		36.15	69.91*	44.50	50.18	6.01	9.81*	7.69	7.83	761.00	461.66	893.33	705.00
In the mean of season													
May King		36.62^{b}	58.73^{b}	49.90^{a}	48.41^{a}	5.33^{b}	7.62^{a}	9.28^{a}	7.41^{a}	738.0^{b}	409.0^{b}	1122.50^{a}	756.50^{a}
Kobak		36.78^{b}	70.17^{a}	48.25^{a}	51.05^{a}	5.37^{b}	9.39^{a}	8.79^{a}	7.85^{a}	861.0^{ab}	579.5^{a}	961.00^{a}	800.50^{a}
Great Lakes		39.50^{a}	64.36^{ab}	38.55^{b}	47.46^{a}	8.46^{a}	8.62^{a}	5.91^{b}	7.80^{a}	1000.3^{a}	595.5^{a}	913.00^{a}	836.16^{a}

Means within the same column followed by the same letter(s) are not significantly different at the probability level of ($p \leq 0.05$) according to Duncan^{a,b} Multiple Range Test

In the autumn experiments we focused on moringa leaf extract (6% MLE) as a plant biostimulant that is safe, inexpensive, and easy to manufacture and apply to plants with the purpose of maximizing quality metrics, yield, and reducing nitrate content. Many research publications describe MLE as an efficient plant biostimulant for improving the quality parameters of many vegetables.

There is also an EU regulation No 1258/2011 for vegetable nitrate content for autumn or winter growing leafy vegetables, with maximum levels permitted for lettuce grown under cover at 5000 mg NO_3^- kg^{-1} and open air at 4000 mg NO_3^- kg^{-1} (Commission regulation, 2011). Thus, our three-year experiment was also repeated in the autumn season but this time under glasshouse weather conditions, where the environmental variable was computerised.

The nitrate content differed significantly in the autumn growing season. Foliar application of 6% MLE performed better in the cooler autumn growing season than in the warmer spring growing season. The mean interaction results shows that there is a significant difference between treated and untreated lettuce cultivars. The use of 6% MLE has the potential to significantly reduce nitrate content by 21%. This reduction was modified based on the genotype reaction and the interaction of the factors with the biostimulant used. The interaction means of "Great Lakes" variety treated with 6% MLE showed the greatest reduction in nitrate content, followed by "May King" and "Kobak" for about 25%, 20%, and 15%, respectively. Despite the fact that the "Great Lakes" variety has a naturally higher nitrate content than "May King" and "Kobak," no significant differences were found among lettuce cultivars (Table 9).

Nitrate accumulation in lettuce cultivars can be influenced by growing years or environmental factors in the same season. For example, in 2019, the "Great Lakes" variety of Crisphead lettuce accumulated significantly more nitrate than the "May King" and "Kobak" varieties. Several studies have found that morphological differences between varieties are related to nitrate accumulation in lettuce, with Crisphead lettuce cultivars accumulating more nitrates than iceberg and romaine lettuce cultivars (EFSA, 2008; Santamaria et al., 1999).

The effect of plant biostimulants on SPAD and NDVI value of lettuce

According to the results in Table 10, there is a negative connection between SPAD and NDVI. Biostimulants increase SPAD while decreasing NDVI and conversely. This indicates that plants with greater nitrogen and chlorophyll content will be subjected to more biotic stress than plants with lower nitrogen and chlorophyll content. This data supports the suggested assumption by Hendry and Price (1993) that a quick increase in total chlorophyll/carotenoids is a known plant stress response. The results reveal that when the plants were treated with biostimulants, there was no significant reduction in the SPAD value. Also, there was a little difference between treated and untreated plants with Bistep and MLE, however the NDVI value was increased. However, as compared to the control plants, Willow and W+B did not significantly improve SPAD and NDVI. Treatments have resulted in a considerable improvement during the course of the growing years. In the growing year of 2021, where there was a substantial light emission during the lettuce growing period, the SPAD value was much higher. Whereas the lowest NDVI value was shown to be significant in the growth year of 2020.

Table 10. Effect of plant biostimulants on the SPAD and NDVI value in different lettuce cultivars grown in **spring** seasons (Debrecen, 2019-2021)

Treatments	SPAD				NDVI			
	2019	**2020**	**2021**	**Mean**	**2019**	**2020**	**2021**	**Mean**
Control	29.86^{ab}	34.05^{b}	33.85^{a}	32.58^{a}	0.730^{a}	0.590^{b}	0.716^{abc}	0.678^{a}
Willow	28.34^{b}	36.20^{a}	32.04^{b}	32.19^{a}	0.713^{a}	0.604^{b}	0.710^{bc}	0.675^{a}
Bistep	26.23^{c}	30.41^{c}	33.95^{a}	30.19^{a}	0.726^{a}	0.662^{a}	0.744^{a}	0.710^{a}
W+B	31.64^{a}	32.03^{c}	33.95^{a}	32.54^{a}	0.708^{a}	0.602^{b}	0.737^{ab}	0.682^{a}
MLE	-	28.25^{d}	33.84^{a}	31.04^{a}	-	0.700^{a}	0.690^{c}	0.695^{a}
Mean	29.01^{b}	32.18^{ab}	33.52^{a}	-	0.714^{a}	0.631^{b}	0.719^{a}	-

Means within the same column followed by the same letter(s) are not significantly different ($P \leq 0.05$) according to Duncan[a,b] test Multiple Range Test

Similar experiments in the autumn season show that the SPAD value or chlorophyll content in lettuce leaves were significantly lower than in the spring season, but there was no difference in the NDVI value between the two seasons (Table 11).

It is obvious that chlorophyll content is directly related to light emission, light quality, and duration (Fu et al., 2012). The light intensity in the growing location varies greatly between spring and autumn seasons; Tables 2 and 4 show a substantial difference in light emission in the experimental location during the lettuce growing seasons. There was also a significant difference in the duration of light between the two growing seasons.

Table 11. Interaction of plant variety, cultivar and growing years on SPAD and NDVI value in lettuce grown in the **autumn** seasons (Debrecen, 2019-2021).

Varieties	Treatments	SPAD value				NDVI value			
		2019	2020	2021	Mean	2019	2020	2021	Mean
Interaction between MLE foliar application and plant variety									
May King	Control	13.16	19.88	17.70	16.91	0.736	0.711	0.716	0.721
	MLE	17.56*	21.71	17.84	19.03	0.753	0.713	0.734	0.733
Kobak	Control	16.23	19.90	17.42	17.85	0.778	0.726	0.726	0.743
	MLE	23.90*	20.45	19.06*	21.13	0.793	0.703	0.748*	0.748
Great Lakes	Control	32.76	37.48	32.72	34.32	0.793	0.663	0.730	0.728
	MLE	40.03*	36.56	34.60*	37.06	0.750	0.676	0.810*	0.745
In the mean of cultivars									
Control		20.72	25.75	22.61	23.02	0.770	0.700	0.720	0.730
MLE		27.16	26.24	23.83	25.74	0.765	0.697	0.762*	0.740
In the mean of season									
May King		15.36^{b}	20.80^{b}	17.77^{b}	17.97^{b}	0.745^{b}	0.712^{a}	0.720^{b}	0.725^{a}
Kobak		20.06^{b}	20.17^{b}	18.24^{b}	19.49^{b}	0.786^{a}	0.715^{a}	0.732^{ab}	0.744^{a}
Great Lakes		36.40^{a}	37.02^{a}	33.66^{a}	35.69^{a}	0.771^{ab}	0.670^{b}	0.770^{a}	0.737^{a}

Means within the same column followed by the same letter(s) are not significantly different at the probability level of ($p \leq 0.01$) according to Duncan[a,b] Multiple Range Test

The light duration in the experimental location was between 8 and 11.5 hours in the autumn growing season, and 11 to 15.5 hours in the spring growing season (Time and Date, 2022). A similar result to the spring growing season was detected for the influence of plant biostimulants, growing years, plant varieties and their interactions on the SPAD value. The means show that plant biostimulant of 6% MLE could slightly improve chlorophyll content, but no significant differences was recorded (Table 11). However, there was a significant difference between lettuce genotypes, with the greener lettuce cultivar "Great Lakes" containing significantly more chlorophyll than the two other varieties "May King" and "Kobak." Furthermore, among the growing years, the interaction effect of plant biostimulants, growing years, and lettuce cultivars was observed. The means, on the other hand, show a slight improvement in the SPAD value, but no statistically significant difference. The interaction factors had the greatest impact during the season, where there was a bit of a similarity from beginning to end with a better light emission from 133.54 to 52.57 (W m^{-2}), whereas the lowest chlorophyll content was detected in the colder growing year of 2021.

The above factors had less of an impact on the normalized difference vegetation index (NDVI) during the autumn season. The NDVI is a non-destructive method for detecting abiotic stress in plants (Sandmann et al., 2018). Our experiments in the autumn season were conducted in a fully controlled environment (glasshouse), so we expected less abiotic stress than growing lettuce in a plastic house in the spring season. Table 11 shows that the NDVI value in the autumn season was slightly higher than in the spring season. This could be due to the fact that there were less unfavourable factors inside the glasshouse, whereas lettuce grown in a plastic house was not fully protected.

Plant biostimulant (6% MLE) performed better in climates with higher light emission during the growing seasons of 2021, whereas the lowest NDVI values were recorded in climates with lower light emission in 2020. However, lettuce cultivars had no significant influence on NDVI, but the differences varied depending on the growing year. While, higher NDVI values were observed during the seasons of lower temperature and higher light intensity in 2021.

Correlation and regression analysis results

Correlation and regression analysis results of the spring experiments

The correlation analysis in Table 12 was applied to the spring experiments of three years' results to see if there was a link between the measured parameters. The nitrate content had significantly negative correlated with lettuce fresh head weight and total polyphenol content at *p* =0.05, with larger head weight containing less nitrate than smaller head weight and plants with higher total polyphenol content containing less nitrate. The correlation between head weight, SPAD, and NDVI, on the other hand, was significantly positive. Vitamin C and SPAD (chlorophyll content), on the other hand, had a positive correlation with nitrate and NDVI at p = 0.01 and p = 0.05, respectively.

Table 12. Correlation analysis for some physical and bioactive compounds of lettuce grown in **spring** seasons (Debrecen, 2019-2021)

Correlations for spring experiments						
Parameters	Head Weight	Total polyphenol	Vitamin C	Nitrate	SPAD	NDVI
Head Weight	1.00					
Total polyphenol	0.087	1.00				
Vitamin C	0.159	0.166	1.00			
Nitrate	-0.234**	-0.234**	0.181*	1.00		
SPAD	0.292**	-0.119	0.077	0.144	1.00	
NDVI	0.585**	0.062	0.231**	-0.047	0.274**	1.00

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed.

Correlation and regression analysis of the autumn experiments

During the autumn season, the correlation between some very important physical and bioactive compounds was different. The only statistically significant relationship discovered was one between total polyphenol and vitamin C content and NDVI value. Total polyphenol content was found to be significantly correlated with vitamin C but negatively correlated with nitrate content and NDVI value. Table 13 shows that increasing vitamin C content had a significant inverse correlation with NDVI value at p = 0.01.

Table 13. Correlation analysis for some physical and bioactive compounds of lettuce grown in **autumn** seasons (Debrecen, 2019-2021)

Correlations for autumn experiments						
Parameters	Head weight	Total polyphenol	Vitamin C	Nitrate	SPAD	NDVI
Head weight	1.00					
Total polyphenol	0.017	1.00				
Vitamin C	0.029	0.647**	1.00			
Nitrate	0.185	-0.541*	0.071	1.00		
SPAD	0.102	0.019	0.112	-0.047	1.00	
NDVI	0.21	-0.732**	-0.567*	0.444	-0.062	1.00

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

The results of the regression analysis were rather complex due to the evaluation of different varieties and different years. We attempted to demonstrate some links between the influence of biostimulants on various bioactive compounds in autumn and springtime using this analysis. Our various analyses revealed a genuine link between vitamin C content evaluation and rising spring temperatures. As can be seen, the biostimulant with the highest regression (R^2) was Vitamin C. Willow has an R^2 of 0.588, Bistep has an R^2 of 0.371, and Moringa leaf extract (MLE) has an R^2 of 0.342. (Figure 9, 10 and 11).

All of the applied plant biostimulants (Willow, Bistep and Moringa leaf extract) supported this trend. However, the regression analyses of other quality parameters were not shown or produced a strong result.

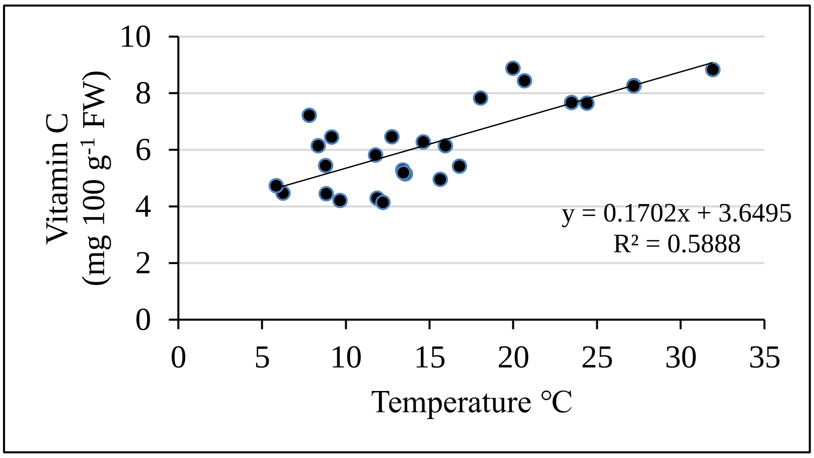

Figure 9. The results of the regression equation for vitamin C vs temperature °C of lettuce growing in three years of experiments in the spring season treated with Willow bark extract (Debrecen, 2019-2021)

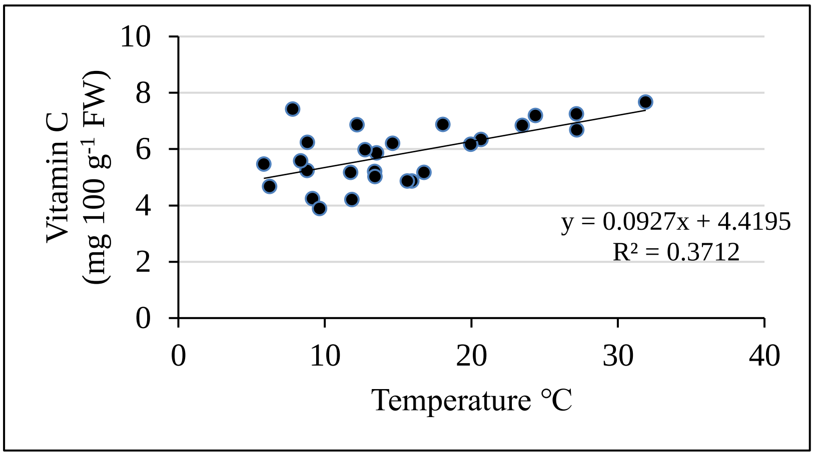

Figure 10. The results of the regression equation for vitamin C vs temperature °C of lettuce growing in three years of experiments in the spring season treated with Bistep plant biostimulant (Debrecen, 2019-2021)

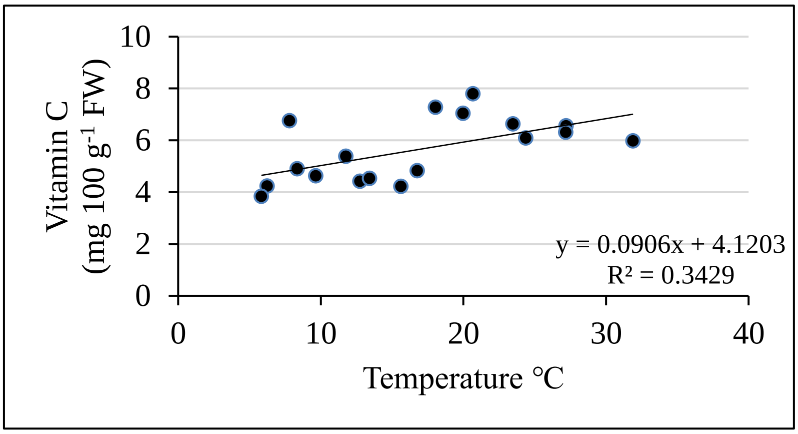

Figure 11. The results of the regression equation for vitamin C vs temperature °C of lettuce growing in three years of experiments in the spring season treated with Moringa leaf extract (MLE) (Debrecen, 2019-2021)

4. NEW SCIENTIFICAL RESULTS

1. Moringa leaf extract (6% MLE) was applied as a plant biostimulant which it could reduce nitrate content in lettuce cultivars grown under glasshouse in the autumn season by 20%, 15%, and 25%, depending on the lettuce cultivar. But this impact was less seen in spring season. This could be because moringa leaf extract has a stronger influence in lower temperature regime than warmer. Bistep (B) plant biostimulant could also reduce nitrate levels in lettuce grown under plastic house in the spring season by 13%. However, using Willow bark extract (W) in the spring season increased the nitrate content by around 20%.
2. Willow bark extract (W) can be used not only as a biofungicide, as many researchers recommend, but also as plant biostimulants to improve some physical quality parameters as head structure and head closing and head weight in “Great Lakes” variety, vitamin C and magnesium content.
3. Combining plant biostimulants could give better results in terms of improving some nutritional value in lettuce without affecting nitrate content than single applications. Willow + Bistep (W+B) could increase the nutritional value of lettuce in terms of macronutrient content (calcium, phosphorus, magnesium, sodium, sulphur), micronutrient content (iron, manganese, and copper), and total polyphenols by about 10%.
4. Lettuce grown in the spring produced higher physical and nutritional value than lettuce grown in the autumn. This was due primarily to environmental factors. Light intensity and duration were higher in the spring than in the autumn, and lettuce is a light-sensitive vegetable. Our recorded metrological data showed that the daily light duration in the spring season was approximately (11 to 15 hours), but in the autumn season was at about (9 to 11 hours).
5. There was also a large variation among the lettuce cultivars. The "Great Lakes" variety produces larger head weight, greater mineral content (potassium, calcium, magnesium, sulphur, copper, manganese and iron) and chlorophyll content. In contrast, greater number of leaves, phosphorus content was found in "May King" and "Kobak" lettuce cultivars in spring and autumn seasons.
6. The interaction of the factors mentioned (genetic, climate, and growing seasons) had a greater impact on physical parameters than bioactive compounds and mineral content. The interaction of Willow bark extract (W) and Willow + Bistep (W+ B) with the "Great Lakes" variety resulted in a significant improvement in head weight. The interaction of moringa leaf extract (MLE) with the "May King" variety led to a significant larger head diameter.

The "Kobak" variety, when treated with moringa leaf extract (MLE), had better head closure than the other varieties.

7. The correlation analysis shows that total polyphenols had a negative correlation with nitrate content in both seasons, for the springtime -0.234**, whereas for autumn time -0.541*. Head weight had a negative correlation with nitrate only in the spring season. The correlation analyses for the other measured parameters varied according to the growing season. Vitamin C had a significant positive correlation with NDVI in the spring season (0.231**), but a negative correlation was found in the autumn season (-0.567*).
8. Regression analysis shows that plant bio-stimulants (Willow, Bistep, and Moringa leaf extract) could only improve vitamin C through increasing temperature in the spring season at R^2 = 0.588, 0.371, and 0.342, respectively.

5. PRACTICAL UTILIZATIONS

1. Based on our three years of research in both seasons (spring and autumn), biostimulants can be more effective when applied to lettuce in a cooler environment with high humidity level.
2. Growing leafy vegetables, particularly lettuce, requires consideration of environmental (light emission and temperature) and plant varieties, so we recommend growing "May King" and "Kobak" varieties primarily in spring, while Great Lakes is best grown in autumn.
3. Willow bark extract (W) is a new biostimulant that can be applied to plants through irrigation once every two weeks at a concentration of 3% to improve other quality parameters in addition to acting as a fungicide and root enhancer.
4. It can be stated that plastic-houses and glasshouses are suitable for lettuce production, however lettuce grown in autumn season may require longer and much intensive light and this can be done with artificial lightening.
5. Our findings suggest that, in addition to the Bistep manufactured biostimulant, home-made biostimulants Willow (W) and moringa leaf extract (MLE) can be applied to lettuce as a safe, easy-to-prepare, and low-cost product with favourable results.
6. Finally, we recommend applying the Willow+ Bistep (W+ B) biostimulant combination to lettuce grown in the spring season in the amounts of 50-70 mL plant^{-1}+ 15-20 mL plant^{-1} in cooler weather conditions of early morning or late afternoon with the doors open to keep the temperature cool and proper air circulation under plastic-house.
7. It is also strongly recommended to apply moringa leaf extract (% MLE) to lettuce growing in autumn as a foliar spray (15-20 mL plant^{-1}) or with irrigation. Because it improves not only some quality parameters but also reduces nitrate content in plant leaves.

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7. LIST OF PUBLICATIONS

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Subject: PhD Publication List

Candidate: Arshad Abdulkhalq Yaseen
Doctoral School: Kálmán Kerpely Doctoral School
MTMT ID: 10081851

List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (2)

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13. **Yaseen, A. A.**, Ahmed, S.: Interaction effect of planting date and foliar application on some vegetative growth characters and yield of broccoli (Brassica olerasea var italica) grown under unheated plastic tunnel.
Journal of Garmian University. 4 (ICBS), 405-418, 2017. EISSN: 2522-3879.
DOI: http://dx.doi.org/10.24271/garmian.151

Total IF of journals (all publications): 9,743
Total IF of journals (publications related to the dissertation): 6,326

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

02 March, 2022