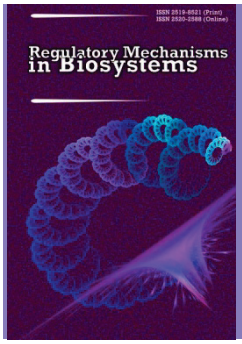

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Effects of humate and neodymium on phytochemical levels in kale at different ontogenetic stages

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The increased global mortality caused by the rise in chronic diseases can be reduced by the consumption of antioxidant-rich foods. Leafy green kale (*Brassica oleracea* var. *sabellica*) has high nutritional value due to its high antioxidant content and its consumption is highly correlated with a reduced risk of developing some chronic diseases. The paper focuses on examining the effects of two different mineral nutrients – neodymium and potassium humate – on the accumulation of flavonoids, vitamin C, phenolic compounds, carotenoids and malondialdehyde (MDA) content in field-grown leafy green kale. Leaves from treated plants were collected at three stages of maturity –14 weeks, 18 weeks, and 22 weeks and subjected to spectrophotometric analysis. The results showed that the stimulatory effect of both mineral nutrients on the phytochemicals varied at the different growth stages - the highest significant effect of neodymium has been mostly observed at week 18 with high flavonoid, vitamin C, and carotenoid levels. Phenolic compounds for this variant are the same as in the control samples, while the level of malondialdehyde was reduced by 21.8%, signifying increased antioxidant activity. The rare earth element primarily maintained some phytochemical content at weeks 14 and 22. Following soil treatment with potassium humate, the levels of studied phytochemical compounds were either maintained or elevated at weeks 14, 18, and 22. Humic acid exerted the greatest decrease in malondialdehyde content in kale at week 14, indicating a reduction in the lipid peroxidation process in leaves. Accordingly, the harvest date of leafy green kale (*Brassica oleracea* var. *sabellica*) should depend on the type of mineral nutrient applied. The obtained results provide information that may be relevant to the production of functional varieties and enhance the nutritional and possibly the economic value of kale.

Keywords: ontogenesis; mineral nutrients; phenolic compounds; vitamin C; carotenoids; malondialdehyde.

Introduction

The worldwide outbreak of chronic diseases is an increasing problem that threatens the world's population today. Almost two-thirds of global mortality is due to these diseases. Increasing evidence suggests that these diseases are associated with lifestyle, stress, and also under-consumption of antioxidant-rich fruits and vegetables (Gakidou et al., 2017; Campbel et al., 2020). This popular understanding of how diet affects diseases has created a market for functional foods, that is, foods that not only satisfy hunger and provide necessary nutrients for humans but also aid in the prevention of or treatment of diseases (Chiu et al., 2018; Santini et al., 2018).

Leafy green kale (*Brassica oleracea* var. *sabellica*) is a popular cruciferous superfood grown mainly in Europe and America and known for its relatively high levels of bioavailable antioxidants that boost the immune system, lower the risk of cancers, preclude oxidative stress, produce detoxification enzymes, mitigate the proliferation of cancer cells, and hinder malignant transformation and carcinogenic mutations in humans (Neugart et al., 2018). Phenolic compounds, vitamin C and carotenoids are major antioxidants in *Brassica* vegetables and contribute more than 80% to their total antioxidant capacity. Among *Brassica* vegetables, kale has been reported to exhibit the highest antioxidant capacity. Phenolic compounds consist of a large group of secondary metabolites widely distributed in the plant kingdom. The health benefits of polyphenols include not only their function as antioxidants but also their participation in gene expression, cell signaling etc. Flavonoids are the largest group of phenolic compounds (Biegańska-Marecik et al., 2017; Michalak et al., 2020). Vitamin C has numerous biological activities in the body and is known to reduce the risk of several diseases (Lafarga et al., 2018). Carotenoids are essential biological antioxidants that are associated with the prevention of cancers, cardiovascular diseases and some degenerative diseases such as macular degeneration (Lee et al., 2020).

Kale is harvested for consumption at different ontogenetic stages as food. Different types and varieties of *Brassica* usually have similar biochemical composition but differ quantitatively (Favela-González et al., 2020). It is common knowledge that the content of these health-benefiting compounds in plants is affected by genetic factors, stage of maturity, and environmental factors (León-Chan et al., 2017; De Masi et al., 2020). Manipulation of these factors can affect the content of flavonoids, vitamin C, phenolic compounds, carotenoids, and malondialdehyde (MDA) (Dias, 2013). It was, therefore, important to estimate the amount of these essential compounds in humate and neodymium-treated kale at different development stages in order to evaluate optimum conditions for increasing the studied phytochemicals.

Rare earth elements are a group of 17 chemical elements in the periodic table which includes neodymium (Nd). Although rare earth elements have been popularly used within the agriculture industry in China for decades, there is a gradual surge in interest in their application to medicinal plants in recent years. Now millions of tons of fertilizers containing rare earth metals are used worldwide to increase crop yields. The use of rare-earth metals on many hectares of cultivated land has shown interesting biological effects on plants such as the germination of seeds, the growth of roots, total biomass, and the production of secondary metabolites. They can cause negative or positive physiological effects on plants depending on the dosage and other conditions. Positive effects and many physiological reactions of plants after treatment with rare earth metals are described in various literature sources (Zhang et al., 2013).

Potassium humate is a commercial product containing many elements necessary for the development of plant life. The application of humic substances is increasingly being accepted in agricultural practice. Its mechanism of possible growth-stimulating effect is usually associated with hormone-like effects, activation of photosynthesis, acceleration of cell division, increased permeability of plant cell membranes and improved absorption of nutrients, and reduced absorption of toxic elements, and improved plant response to salinity.

In addition, potassium humate can be used as a cheap source of potassium and a soil dressing, watering, or foliar dressing. Humic acid (HA) is one of the primary components of humate. The use of HA has several advantages, and agronomists worldwide accept it as an integral part of their fertilizer program. Researchers have found that foliar application of humic acid leads to a positive effect on plant growth and improved productivity of the garlic plant (Canellas et al., 2015; Mohsen et al., 2017).

Considering the current increased cost of healthcare and the appreciation that lifestyle habits along with dietary patterns constitute modifiable risk factors related to cancer, coronary diseases, diabetes and osteoporosis, studying the effect of neodymium and potassium humate on the accumulation of bioactive compounds in kale is highly promising since the concept of functional food development or production is aimed at increasing the contents of beneficial functional ingredients like phenolic compounds, flavonoids, vitamin C, carotenoids etc. in the diet so that they are more available and accessible to the consumer.

This experiment aimed to investigate how neodymium and humate affect the accumulation of flavonoids, phenolic compounds, vitamin C, carotenoids, and MDA in kale at different stages of maturity. To achieve the set goal, we set the task to determine changes in the content of the phytochemicals mentioned above in the leaves of neodymium and humate-treated kale plants, harvested at 14, 18, and 22 weeks of growth.

The obtained data can be implemented as a guideline for acquiring the most significant number of desirable metabolites and increasing the economic benefits of using and consuming this type of cabbage.

Materials and methods

The plants were sown in the fields in July 2019. At the stage of two to three leaves, the mineral nutrients humate (K – salts of humic acids and microelements) and neodymium (Nd 10^{-5}) were applied to the soil. Control variants were treated with water. The edible matured kale leaves were harvested at weeks 14, 18, and 22 to determine the content of total flavonoids, Vitamin C, total phenolics, total carotenoid, and MDA. Leaves were transported from the field to the laboratory in liquified nitrogen and stored at –80 ºC until analysis. Analyses were performed on spectrophotometer UNICO 2800 (Russian Federation and registered under No. 38106-08, and a certificate of the State Standard of the Russian Federation No. 32007).

Phenolic compounds were determined by a modified Folin–Ciocalteu method at a wavelength of 725 nm on a spectrophotometer. Aqueous extraction of phenolic compounds from kale leaves was carried out by incubating 50 mg of air-dried crushed leaves, which were placed in 1.5 mL Eppendorf tubes with distilled water at 70 ºC for 45 minutes. The extract was centrifuged at 15,000 rpm for 5 minutes. The supernatant was decanted into a 1.5 mL Eppendorf tube. Distilled water (0.5–0.7 mL) was added to the sediment, shaken and centrifuged again under the same conditions. The supernatants were pooled, and the final volume was adjusted to 1.5mL. The extract was kept in the dark at 4 ºC for subsequent determination of the total amount of phenolic compounds.

To determine the total amount of phenolic compounds, 75 mL of the extract was placed in 1.5 mL test tubes, 75 mL of Folin-Denis reagent was added and mixed. After 3 minutes, 120 mL of 10% saturated $NaHCO_3$ was added, mixed, 1.2 mL of distilled water was added, and after 45 minutes, it was centrifuged at 16000 rpm for 2 minutes. Next, the optical density of the supernatant was measured at a wavelength of 725 nm using a UNICO-2800 spectrophotometer. For the reference solution, 75 µL of solvent was used instead of the extract. The total content of soluble phenolic compounds in the studied variants was calculated by the formula:

$$C = E * K * R * V/m * 1000,$$

where C – concentration of phenolic compounds (mg/g dry weight of the test sample), E – optical density at 725 nm, K – the conversion factor for the reference substance – (-) epicatechin (480); R – dilution, times; V – extract volume (mL), m – the weight of the sample of plant material (grams) (Tandoro et al., 2020).

Using the colourimetric method at 420 nm, we calculated the total flavonoids content by their reaction with $AlCl_3$. To assess the content of flavonoids in kale cabbage, alcohol extraction was performed. For this, 1g of kale cabbage leaves was placed in a flask with a thin section with a capacity of 150 mL, and 30 mL of 90% alcohol containing 1% concentrated HCl was added. The flask with a reflux condenser was heated in a boiling water bath for 30 minutes. The flask was then cooled to room temperature and the contents were filtered through filter paper into a 100 mL volumetric flask. The extraction was repeated once more as described above, then once more with 90% alcohol for 30 minutes. The extracts were filtered through one filter into the original flask. To wash the filter, 90% alcohol was used to bring the filtrate to the mark. This solution is designated as solution A. 2 mL of solution A was placed in a 25 mL volumetric flask, 1 mL of a 1% solution of aluminium chloride in 95% alcohol was added and the volume of the solution was brought to the mark with 95% alcohol. After 20 minutes, at a wavelength of 430 nm, the optical density of the solution was measured on a UNICO-2800 spectrophotometer. For comparison, we used a solution consisting of 2 mL of solution A, brought to the mark with 95% alcohol in a 25 mL volumetric flask. The content of the sum of flavonoids in terms of quercetin and absolutely dry raw materials in percent (X) was calculated by the formula:

$$X = (D*25*100*100*100)/(764.6*m*2*(100–W)),$$

where D is the optical density of the studied solution; 764.6 – specific indicator of adsorption of a complex of quercetin with $AlCl_3$ at a wavelength of 430 nm; m – mass of vegetable raw materials (g); W – the weight loss during drying of raw materials (%) (Makuasa & Ningsih, 2020).

MDA content level has been assessed by the amount of accumulated product formed due to its reaction with thiobarbituric acid. The formation of malondialdehyde (MDA), a secondary end-product of the oxidation of polyunsaturated fatty acids, is considered a useful indicator of total lipid peroxidation. 0.5 g of leaves were ground in 1.5 mL of 20% TCA. The homogenate was centrifuged at 12000 g for 5 minutes. The obtained supernatant material was used as a sample for analysis. Then the supernatant was added to two tight-fitting tubes, 1 mL each. To one of the samples, 1 mL of 20% TCA was added; later this sample was used for spectrophotometric measurements as a control. To another sample, 1 mL of 0.5% TBA solution was added. For 30 min, the samples were incubated in a boiling water bath at a temperature of 100 ºC and then cooled at room temperature. This was measured on a UNICO-2800 spectrophotometer at two different wavelengths of 532 and 600 nm to correct nonspecific absorption. Calculation formula:

$$C = [[\Delta D/155]*X*V]/[[m*\Delta m]*1],$$

where C is the amount of MDA (mmol/g dry weight); ΔD is the difference in optical density of the sample at 532 and 600 nm; 155 – extinction coefficient of MDA at 532 nm, $mM^{-1}cm^{-1}$; X – dilution, i.e. the total volume of the reaction mixture was divided by the amount of the introduced extract sample; V – the volume of the extract (mL); m – the mass of the wet sample (g); Δm – the ratio of dry and wet weight; 1 – optical path length (cm) (Anteh et al., 2021).

The level of vitamin C content was calculated as the total of dehydroascorbic acid plus ascorbic acid by spectrophotometry. A weighed sample of raw 500 mg kale cabbage leaves was ground in 1 mL of a buffer solution of ammonium citrate (pH = 3.69), centrifuged for 5 min at 12500 g. The supernatant was used to assess the accumulation of vitamin C in the leaves of kale. To 0.5 mL of the extract we added 25 µL of 1% $K_3[Fe(CN)_6]$ solution, 25 µL of 2% NaF solution and incubated for 5 minutes. Then 1.9 mL of distilled water and 50 µL of 2% $FeCl_3$ solution were added. The resulting solution was kept for 5–7 minutes with occasional shaking. Then a spectrophotometric analysis was carried out. Optical density was measured at 680 nm. A reaction mixture with a buffer instead of an extract was used as a control solution. The concentration of vitamin C was determined using a calibration curve. The vitamin C content was calculated using the formula:

$$C = (K*V*X)/(m*\Delta m*L),$$

where C is the content of vitamin C (µg/g wet weight); K – the concentration of vitamin C (µg/mL); V – the total volume of the extract (mL); X –

the dilution of the extract in the reaction mixture; L – the length of the optical path (cm); m – the mass of the wet sample (g); Δm – the ratio of dry weight to wet weight (Anteh et al., 2021).

The content of carotenoids was calculated using the spectrophotometric method. 0.2 g of the leaves were placed in a porcelain mortar and a little calcium dioxide, washed quartz sand and triturated with 2 mL of 80% solution were added. To the crushed mass, 4 mL of acetone was added and ground again for several minutes. The extract was carefully poured into a funnel with a paper filter. The filter was washed several times with acetone. Then the filtrate was poured into a 10 mL volumetric flask and the contents of the flask were brought to the mark, closed with a rubber stopper, and then thoroughly shaken. Then the resulting solution was used to determine the content of carotenoids. The measurements were carried out on a UNICO-2800 spectrophotometer at three wavelengths: 665, 649 and 452.5 nm. The concentration of pigments is calculated using the equations:

$$C_{chla} = 11.63 * D_{665} - 2.39 * D_{649};$$
$$C_{chlb} = 20.11 * D_{649} - 5.18 * D_{665};$$
$$C_{car} = 4.2 * D_{452.5} - [0.0264 * C_{chla} + 0.426 * C_{chlb}],$$

where D – experimentally obtained values of optical density at the corresponding wavelengths; C_{car}, C_{chla} and C_{chlb} – respectively, the sum of the concentration of carotenoids and the concentration of chlorophylls a and b (mg/L). To determine the content of carotenoids in plant materials (mg/g) of wet weight was calculated by the formula:

$$M = C * (V/P),$$

where M is the pigment content in plant raw materials (mg/g); C – the concentration of pigments (mg/L); V – the volume of the pigment extract (mg/L); P – the sample of plant material (grams) (Anteh et al., 2021).

Experiments were conducted in 5 biological repeats. Statistically, the processing of data was conducted using GraphPad prism v.8.4.3. The reliability of the difference was determined by ANOVA test at $P < 0.05$.

Results

Flavonoid content in humate treated kale at week 14 was significantly higher than in the neodymium treated samples. Flavonoid content in plants treated with humate increased by 13.0%, while no change occurred in plants with neodymium treatment. Although there was an overall decline in flavonoid content at weeks 18 and 22, neodymium at week 18 boosted flavonoid content by 15.9% in comparison with control samples. Humate increased flavonoid content by 27.4%, whereas there was no significant increase in samples treated with neodymium at week 22. Humate treated kale leaves contained higher levels of flavonoid at all three stages of maturity (Fig. 1).

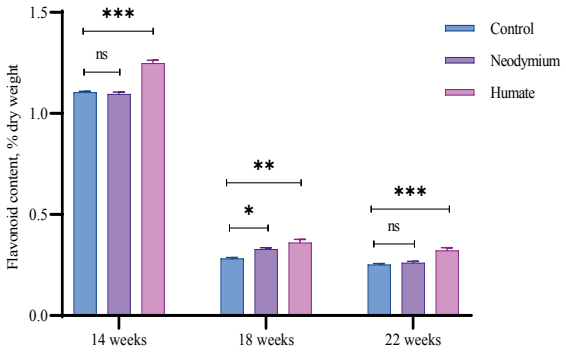

Fig. 1. The influence of mineral nutrition on the content of flavonoids in plants *Brassica oleracea* var. *sabellica* in ontogeny: ns, *, ** and *** represent $P > 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively

In Figure 2, the application of both mineral nutrients stimulated a significant decline in the phenolic compound content at week 14. No significant change was registered in phenolic compound content for the experimental variants at week 18. At week 22, humate treatment raised the level of phenolic compounds by 76.0%, an effect higher than observed in kale plants treated with neodymium, which induced a 23.6% increase.

Vitamin C content was generally higher in leaves harvested at weeks 14 and 22. Only humate treatment augmented vitamin C content by 24.3% at week 14, however at week 18 neodymium increased vitamin C levels in kale by 36.0%, approximately three-fold the effect induced by humate (13.4%). Although at week 22 vitamin C accumulation in kale leaves rose for all variants, a slight but significant increase of 2.5% was observed in the humate samples (Fig. 3).

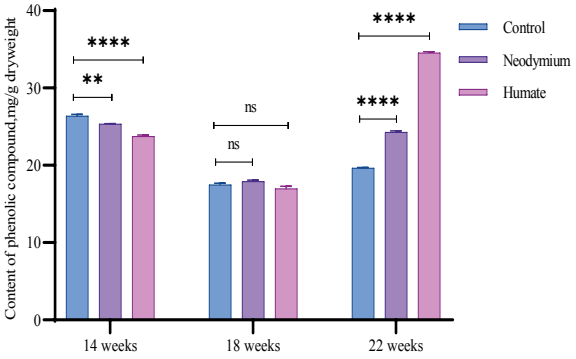

Fig. 2. The influence of mineral nutrition on the content of phenolic compounds in plants *Brassica oleracea* var. *sabellica* in ontogeny: ns, ** and ****, represent $P > 0.05$, $P < 0.01$ and $P < 0.0001$, respectively

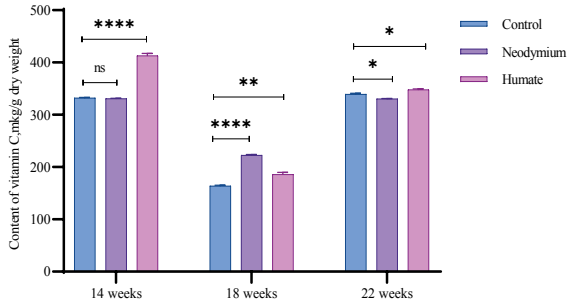

Fig. 3. The influence of mineral nutrition on the content of vitamin C in plants *Brassica oleracea* var. *sabellica* in ontogeny: ns, *, ** and ****, represent $P > 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.0001$, respectively

At week 14, carotenoid levels were significantly elevated in neodymium treated kale leaves by 100%, whereas the accumulation of carotenoids in humate treated variants was similar to control samples, but at week 18, carotenoid levels for neodymium and humate-treated kale were similarly enhanced by 42.8% and 45.1%, respectively. At week 22, the accumulation of carotenoids in kale leaves induced by both mineral fertilizers was not significantly different from control samples (Fig. 4).

The amount of MDA measured in neodymium and humate treated kale was respectively 56.0% and 56.6% lower than the control samples at week 14. Data in Figure 5 indicates that the addition of neodymium and humate significantly decreased MDA by 21.8% and 36.2%, respectively, compared with control at 18 weeks. The application of neodymium to kale significantly stimulated a 42.3% decline in MDA biosynthesis, whereas treating kale with humate did not cause any significant change in MDA at this stage of growth (Fig. 5).

Discussion

A recent increase in consumer interest in natural sources of health benefiting phytochemicals makes manipulating some of the factors that influence the content of these molecules in kale a very promising research area. We set an aim to analyze the effects of neodymium and humate on the content of bioavailable phytochemicals such as flavonoids, vitamin C, phenolic compounds, carotenoids, and malondialdehyde (MDA) in kale (*Brassica oleracea* var. *sabellica*) at different growth stages. The findings of this research reveal that, although kale is a rich source of antioxidants that can minimize the risk of or possibly help cure life-threatening diseases like cancer, diabetes, and heart diseases, the level of accumulation of these antioxidants depends on the type of fertilizer used and the growth stage (time of harvest). It was observed that at the three edible mature stages at weeks 14, 18, and 22, the content of studied phytochemicals in kale (*Brassica oleracea* var. *sabellica*) varied with neodymium and humate treatments.

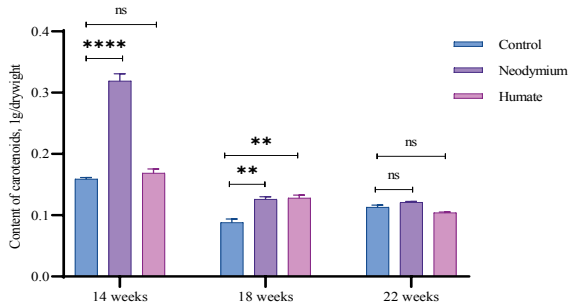

Fig. 4. The influence of mineral nutrition on the content of carotenoids in plants *Brassica oleracea* var. *sabellica* in ontogeny: ns, ** and ****, represent P > 0.05, P < 0.01 and P < 0.0001, respectively

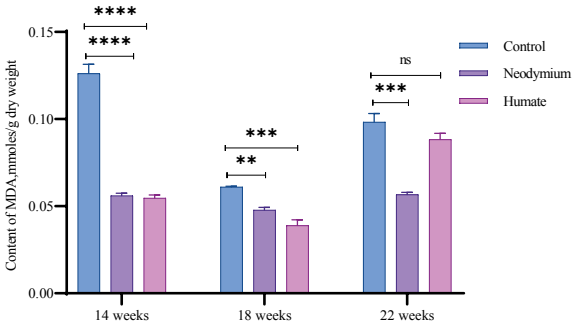

Fig. 5. The influence of mineral nutrition on the content of MDA in plants *Brassica oleracea* var. *sabellica* in ontogeny: **, ***, ****, represent P < 0.01, P < 0.001, and P < 0.0001, respectively

The rare-earth element, neodymium, commonly found in granites, pegmatites, gneisses, and other rocks, is an effective fertilizer for improving plant nutrition when used in small amounts (Zhang et al., 2013). The higher content rise in carotenoids observed at the 14th week and in flavonoids, vitamin C, phenolic compounds, and carotenoids at week 18 in neodymium treated kale leaves compared to humate treated samples (Fig. 6) can be attributed to the low concentration of neodymium used in fertilizing the kale plants. These findings are consistent with previous research works by Rezae et al. (2018) and Chen et al. (2015), which attribute low concentrations of rare-earth elements to positive effects on plant metabolite production.

On the other hand, excessive concentrations adversely alter photosynthesis and antioxidant activity. The highly augmented carotenoid levels are consistent with research findings that suggest that rare-earth elements at low concentrations promote chlorophyll content and the rate of phosphorylation (Fan et al., 2020). Malondialdehyde (MDA) is a lipid peroxidation marker (Tounekti et al., 2011). The treatment of kale with neodymium significantly lowered MDA production in kale (*Brassica oleracea* var. *sabellica*) at all studied stages of development, adducing that neodymium supplements improved the antioxidant system by scavenging reactive oxygen species (ROS) (Shahnaz et al., 2011). The current experiment demonstrates that levels of antioxidants such as flavonoids, vitamin C, phenolic compounds, and carotenoids were either maintained or boosted at all three growth stages.

Different ontogenic stages may affect the concentration of various primary and secondary plant metabolites. The level of production of metabolites is not constant in plants; instead, it changes in different environmental conditions according to the plant's needs. The leaf harvesting times fell within three different seasons; 14 weeks – autumn, 18 weeks – early winter, and 22 weeks – winter. The temperature could have affected the phytochemicals studied. However, further research is needed (Verma & Shukla, 2015).

Humic acids, a precursor of humic compounds, improve the qualitative and quantitative characteristics of plants. The diversity of functional groups contribute special features for humic compounds that stimulate plant growth through carbon induction and carbon metabolism, which as a result, contributes to the synthesis of intermediate compounds that act as precursors for secondary plant metabolism. It can explain the relatively positive effect on the content of flavonoids and vitamin C in kale at all investigated growth stages (Khorasaninejad et al., 2018; Pott et al., 2019). Humic substances in recent research have been found to augment the production of the phenylalanine/tyrosine ammonia lyase (PAL/TAL), which subsequently catalyzes the initial primary step in phenolics biosynthesis by the conversion of tyrosine to p-coumaric acid and phenylalanine to trans-cinnamic acid. PAL/TAL expression is followed by the aggregation of phenol in leaves (Canellas et al., 2015). This factor may explain the 27.4% and 76.0% increase in flavonoids and phenolic compounds in humate-treated kale at week 22, as well as the significant augmentation of flavonoids observed at weeks 14 and 18 in comparison to their control samples. However, at week 14, humate did not have an increasing effect on the level of phenolic compounds, which may be associated with the fact that the plant was at a developmental stage in its life cycle that affected the phenolic compound content (Verma & Shukla, 2015); nonetheless, an exponential rise was registered as the plant grew. The importance of the non-enzymatic antioxidant defence system which consists of ascorbate, carotenoids, phenols, etc., has been exhibited during drought stress. Humic acid stimulates the production of compounds linked to the shikimic pathway that is responsible for this non-enzymatic antioxidant defence (Canellas et al., 2015; Sales et al., 2015). This aspect is illustrated by the favourable effect of humate on vitamin C and carotenoid content of kale gathered at all three investigated harvest times.

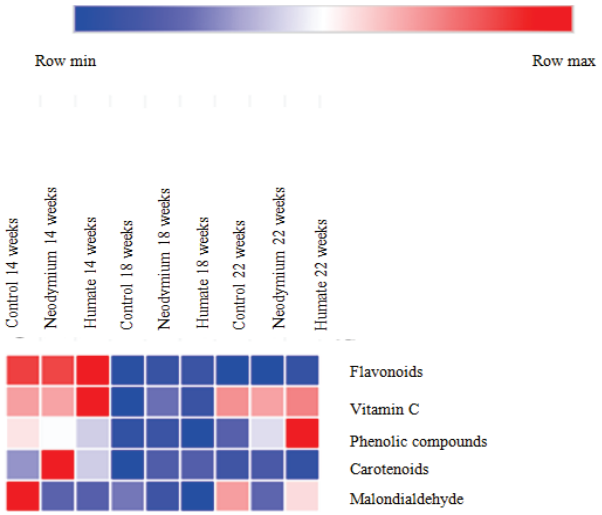

Fig. 6. Heatmap of the percentage increase of phytochemicals studied in kale *Brassica oleracea* var. *sabellica* (L.): red fields indicate the increasing effect of mineral nutrients, whereas blue fields represent the declining effect of the respective mineral nutrient applied

Cold air temperature is a potential drought stress condition that can lead to reactive oxygen species generation and consequently lipid peroxidation (Hasanuzzaman et al., 2012). Humic acids increase resistance to stress by stimulating the antioxidant defence systems, thereby lowering the amount of MDA formed (Shahnaz et al., 2011). In the current experiment, however, the lipid peroxidation marker was lower at 14 weeks (autumn) by 56.6% decrease, at 18 weeks (early winter) by 36.2% decrease and 22 weeks (winter) by 10.1% decrease, which could be a result of the cumulative effect of other abiotic factors in the open field where the plants were cultivated.

At different stages of maturation, the effects of both neodymium and humate on the metabolite content varied (Bauddh & Singh, 2015; Liebelt et al., 2019). Therefore, it is recommended that the harvest date of kale cabbage should depend on the type of fertilizer used in order to procure a more valuable product.

Conclusions

The type of mineral fertilizer applied to kale cabbage variably affected the studied phytochemical content at different maturation stages. Humate positively affected the studied phytochemicals at the earliest and

latest harvest dates, and Neodymium was significantly effective mostly at week 18. The effectiveness of both mineral nutrients in the accumulation of studied biochemical compounds varied at different ontogenetic stages. Although it is an essential agricultural practice for the enhancement of plant growth and maintaining crop yields, the application of mineral nutrients does not solely optimize plant productivity. The current findings allow us to recommend that the choice of harvest time for kale cabbage should depend on the type of utilized fertilizer to procure the most valuable product.

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