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Biochar influences on phytochemical composition and expression genes of curly kale at different treatment times

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Abstract: This study examined the effect of biochar application to the soil at different times on the phytochemical composition (phenolic compounds, carotenoids, vitamin C, sugars, proteins, MDA, and antioxidant activity) and expression of HCT, F3'H and CHS genes (which are involved in the accumulation of phenolic compounds) in cabbage kale (*Brassica oleracea* L. *var. acephala*).
Biochar was prepared from chicken manure, using the method of rapid pyrolysis at a temperature of 400 °C. The retention time at the maximum temperature was 4 h, and the heating rate was 10 °C min^{-1}. The biochar fertilizer was applied to 5–7 day-old kale seedlings at planting in one variant, and in the second experimental variant it was added 1 month after the seedlings were sown.
The results demonstrated that the biochar had a greater effect on the content of phenolic compounds, flavonoids, carotenoids, antioxidant activity, and sugars on the 16-week-old plants when it was added at the time of planting.
Early biochar treatment stimulated an 80-fold increase in HCT gene expression, a 17-fold increase in F3'H gene expression, and a nearly 22-fold increase in CHS gene expression. Late biochar treatment stimulated a roughly 22-fold increase in HCT gene transcripts, a 23-fold increase in F3'H gene transcripts, and a 16-fold increase in CHS gene transcripts in kale. Thus, these experiments once again convincingly prove that treatment of kale with biochar during planting is more effective in stimulating the production of health-promoting compounds.

Key words: *Brassica oleracea* L. *var acephala*, biochar, phytochemical composition, HPLC, expression genes

1. Introduction

Produced by the pyrolysis of a solid organic biomass residue, biochar improves soil quality and promotes plant development and agricultural productivity (Awad et al., 2018). The introduction of biochar fertilizer as an agronomic tool has significantly increased crop yield as per many scientific reports. Biochar's liming effect, high water holding capacity, and capability to increase crop nutrient might be the main factors behind the positive effects. Biochar can effectively sequester the soil carbon for hundreds to thousands of years (Lehmann et al., 2015), and it can mitigate the agricultural emission of greenhouse gases and can be an effective option for waste management. The porous structure of biochar contributes to the improvement of water-retaining properties, aggregate stability, and arability of soils. Biochar also serves as a source of nutrients for plants and creates favorable conditions for the development of beneficial microorganisms when applied to the soil (Hossain et al., 2019). According to the studies of Tian et al. (2016), the addition of biochar obtained from pine wood in the amount of 6 t/ha to the soil increased the content of total soil carbon up to 50%. Since biochar mainly has pH values in the range of 8–10, its introduction into the soil increases the pH values, thereby neutralizing acidic soils (Song et al., 2023). Experimental results of the effect of biochar on plant growth and crop yield are highly variable based on soil type, crops grown, biochar source, and system of farming (Lehmann et al., 2015). Biochar has been known to affect many biochemical and physiological aspects of plants (Atkinson et al., 2010; Semida et al., 2019). The effect of biochar on the synthesis of bioactive compounds varies according to how much concentration is added to the soil (Regmi et al., 2023). Viger et al. (2015) prove that biochar affects the pathway of secondary metabolites by the regulation of genes.

Kale (*Brassica oleracea* L. var. acephala), which is commonly cultivated in Central and Northern Europe and North America, belongs to the family Brassicaceae. It has higher concentrations of phytochemicals, such as glucosinolates (GSLs), phenolic compounds, anthocyanins, amino acids, vitamins, and minerals.

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These health-promoting phytochemicals have both anticarcinogenic and antioxidant properties, and they play an important role in the interaction between plants and their environment, e.g., as feeding deterrents, pollination attractants, compounds protecting against pathogens or various abiotic stresses, antioxidants or signaling molecules (Šamec et al., 2019). According to Cao et al. (1996), kale had the second strongest antioxidant activity against peroxyl radicals among 22 common vegetables, including spinach, broccoli, carrot, and potato.

Various types of organic waste can serve as raw materials for biochar. This includes plant litter, wood, sewage sludge, animal and poultry waste, etc. (Hassan et al., 2020). Biochar obtained from wood waste has a large specific surface area and carbon content, but at the same time a lower content of oxygen and mineral components compared to biochar obtained from manure (Weber et al., 2018). Thus, manure and bird droppings may be more promising substrates for nontraditional fertilization due to their higher nitrogen content.

Most of the current research on biochar's impacts on plant production has focused on quality, stress tolerance, and yields. Very few studies have examined the influence of biochar rates on phytochemical production in plants. Furthermore, there is no research available on how the phytochemical compositions of kale cabbage are affected by biochar application at different times and its effect on the expression of HCT, F3'H, and CHS genes.

We hypothesized that the application of biochar can increase the phytochemical production of kale and stimulate expression of some genes which are responsible for the accumulation of phenolic compounds. Therefore, the objective of this study was to evaluate effects of biochar applications at different times on the photochemical composition and expression of HCT, F3'H, and CHS genes in kale cabbage (*Brassica oleracea* L. var acephala).

2. Materials and methods

2.1. Materials and sample preparation

The study was conducted from January 10 to August 20, 2022, in the plant physiology laboratory of Kazan Federation University in Tatarstan, Russia. The treatments consisted of 3 groups: a control group, a group comprised of kale plants fertilized at planting, and a group comprised of kale plants fertilized 1 month after planting.

The material investigated consisted of fresh kale (*Brassica oleracea* L. var. acephala) leaves. Kale seeds germinated for 5–7 days in Petri dishes on wet filter paper. Then, 45 kale plant seedlings were sown for subsequent analysis. In each treatment, 5 pots were taken and 3 seedlings were sown in each pot. Fifteen samples for each of the 3 variants were cultivated in pots under laboratory conditions. All pots were filled with 500 g of dry sandy-loamy soil.

Soil acidity was neutral with pH = 6.9. The organic matter (humus) content was 1.96%, nitrate nitrogen was 35.5 mg/kg, ammonia nitrogen was 11.3 mg/kg, the mobile phosphorus content was 584 mg/kg; the amount exchangeable calcium was 13.25 mmol/100g and exchangeable magnesium was 1.5 mmol/100g.

The biochar fertilizer was applied to 5–7 day-old kale seedlings at a rate of 10 g/kg at planting in one variant. In the second experimental variant, biochar fertilizer was added at the same rate 1 month after the seedlings were sown. Control seedlings were exempted from fertilizer application.

Kale leaves from the control and experimental samples were harvested at 12 and 16 weeks after planting and immediately fixed in liquid nitrogen and stored at –80 °C for phytochemical analyses.

Biochar was prepared from chicken manure in an industrial pyrolysis plant in the city of Naberezhnye Chelny (the Republic of Tatarstan, Russia), using the method of rapid pyrolysis at the temperature of 400 °C. The retention time at the maximum temperature was 4 h, and the heating rate was 10 °C min^{-1}. Content element: C 18.64%, Corg 7.23%, N 1.88%, P 1.51%, K 1.77%.

Methods for the determination of total content of carbon, organic carbon, nitrogen, potassium, and phosphorus was discussed in detail in a previous paper for Kazan Federation university (Kryntsev et al., 2019).

2.2. Chemical determination by spectrophotometric method

Phenolic compounds were assessed based on levels of gallic acid by the modified Folin–Ciocalteu method, and optical density was measured at 725 nm (Shahidi et al., 2015). Flavonoids were determined by reaction with AlCl3 at 420 nm (Naczk et al., 2004). Vitamin C content was calculated as the sum of ascorbic acid (AA) and dehydroascorbic acid (DHAC) at 680 nm (Sokolovsky et al., 1974). The amount of provitamin A in the acetone extract was calculated using the Wettstein formula at several wavelengths: 662 nm, 644 nm, and 440.5 nm (Vorobyov et al., 2013). The antioxidant activity of the kale leaves was verified based on their ability to inhibit adrenaline autooxidation in vitro, and thereby prevent the formation of reactive oxygen species at a wavelength of 347 (Sirota, 2011). Protein content was determined according to the Lowry method at a wavelength of 750 nm (Peterson, 1979). The sugar content was analyzed using the anthrone method and the optical density was measured at a wavelength of 780 nm (Timofeeva, 1998). The MDA content was evaluated based on the level of accumulation of the product resulting from the reaction which occurred between malondialdehyde and thiobarbituric acid. Measurements were taken at 532 nm, and then again at 600 nm to correct for nonspecific absorption (Dedei et al., 2021).

2.3. The determination of the phenolic compounds content by high-performance liquid chromatography (HPLC)

Alcohol extraction of secondary metabolites was carried out in 70% ethanol on a water bath for 90 min. After obtaining the extract, the identification of phenolic compounds was performed by a BIO-RAD high-pressure chromatographic system (USA). An original column SN-421001911, 5 μm, 4 × 250 mm (USA) was used. Peaks were detected using a dual-wavelength UV HPLC detector BioLogic Quad TecUV-Vis (USA) at 280 and 360 nm. Separation was performed in isocratic mode using a mixture of acetonitrile and water with 1% glacial acetic acid added as the mobile phase at a ratio of 25:75. The eluent flow rate was –0.4 mL/min. A 20 μL sample was added to the column. Chromatography was performed at room temperature (25 ± 2 °C). For the identification of peaks detected on the chromatogram, working standard samples quercetin, isorhamnetin, kaempferol, coumaric acid, benzoic acid, ferulic acid, caffeic acid, synapic acid, and rutin were used.

2.4. Isolation of RNA for real-time PCR and sequential expression analysis of HCT, F3'H, and CHS genes

For total RNA isolation, cDNA synthesis, and expression analysis of HCT, F3'H, and CHS genes, all primers used for the analysis of the gene expression of the respondent genes were developed using the online Primer Blast software [https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi]. In the creation of the primer pair, the size of the amplified fragment and the annealing temperature of the primers for HCT, F3'H, and CHS genes, which are involved in the accumulation of phenolic compounds in cabbage kale, were considered. The kale leaves were ground in liquid nitrogen. A portion of the grounded mass of 100 mg was transferred into 1 mL of ExtractRNA total RNA isolation solution (Eurogen, Moscow, Russia) and incubated at room temperature for 15 min. Then the lysate was centrifuged for 10 min at 14,500 rpm in a Mikro 200R centrifuge (Hettich, Germany).

The supernatant was then transferred into a new sterile test tube and 0.2 mL of chloroform was added, stirred, and centrifuged again at 14,500 rpm in a minispin centrifuge (Eppendorf, Germany) for 10 min. The upper aqueous phase was transferred to a new tube, 0.5 mL isopropanol was added, stirred, and incubated at room temperature for 10 min. The mixture was centrifuged at 12,000 rpm at room temperature for 10 min. The precipitation was washed with 75% ethanol solution, dried, and dissolved in RNase-free water. RNA concentration was measured using a NanoPhotometer NP80 spectrophotometer (Implen, Germany). Prior to utilization, all isolated RNA samples were adjusted to a concentration of 100 ng/mL.

Gene expression was determined by the number of copies produced by mRNA (Cao and Grima, 2020). The OneTube RT-PCRmix reagent kit (Eurogen, Russia), which allows for cDNA synthesis and amplification in a single tube, was used. This process employed primers that had been developed for the HCT, F3′H, and CHS sequences of the cabbage kale genes. The reaction was conducted in a 20 μL volume containing 10 μL qPCRmix – HS SYBR + High ROX (Evrogen), 0.25 мM of each primer, and 1μL of cDNA. Reactions were carried out in 3 repetitions according to the protocol: 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The β-actin gene was used as the housekeeping gene (Li et al., 2021). "Double delta Ct" (DDCt) was calculated after comparing the relative expression levels of PR genes with β-actin (Rao et al., 2013).

2.5. Statistical analysis

The experiments were carried out in five biological replicas. The figures show the values of the mean and standard deviations. Statistical data processing was implemented using GraphPad Prism software version 8.4.3. Significance levels were set at $p \leq 0.05$ and differences among means were determined using a Tukey's HSD test.

3. Results

3.1. Effect of biochar on antioxidant and nutritional properties of kale

The effect of biochar on the phytochemical composition of kale was analyzed. Biochar was applied simultaneously with planting or 1 month (4 weeks) after planting. Plants were analyzed 12 and 16 weeks after planting.

According to our results, the content of phenolic compounds increased under the influence of biochar at both growth stages. At week 12, the level of phenolic compounds was increased by 51% with the plants fertilized at planting and by 23% with a later application of biochar, compared with control (Figure 1A). At week 16, the indicator was increased by 51% preplanting fertilization and by 16% with postplanting fertilization.

Preplanting fertilization in 12-week-old plants resulted in a 44% increase in flavonoid content, while plants with postplanting fertilization showed no significant change in flavonoid amounts. At week 16, biochar induced an increase in flavonoid levels for both variants, with the plants fertilized at planting by 28.5 %, and those with a later application of biochar by 15%, compared with control (Figure 1B).

After 12 week, the plants that were fertilized at planting had a 45% lower vitamin C content than the control plants, while biochar applied 1 month after planting caused no significant changes in vitamin C content. This trend persisted at a later stage of plant development (16 weeks) (Figure 1C).

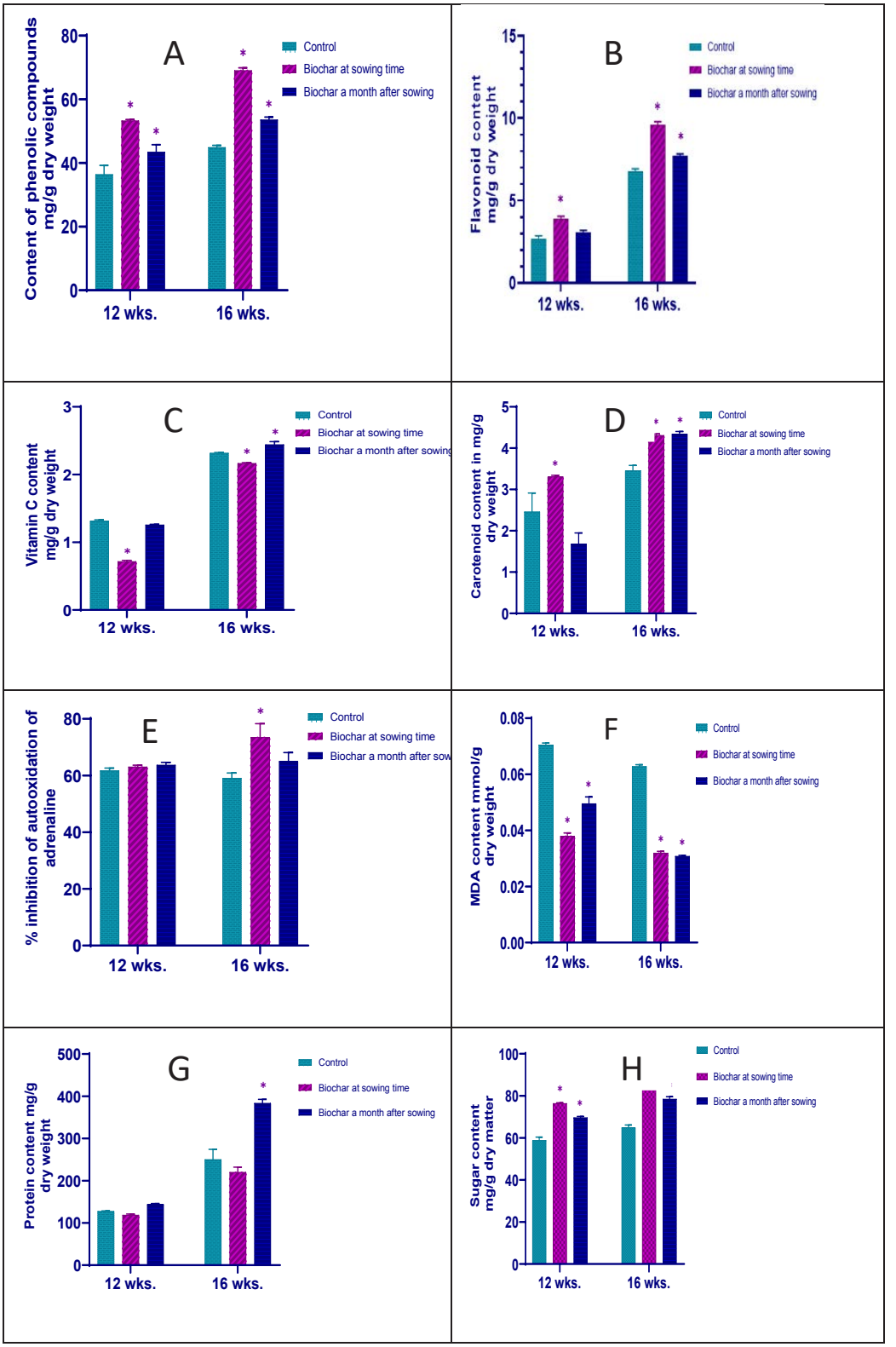

Figure 1. The effect of biochar on the content of: A-phenolic compounds, B-flavonoids, C- vitamin C, D-carotenoids, E- total antioxidant activity, F-MDA, G-proteins, H-sugars in kale plants (*Brassica oleracea* L *var. acephala*) at 12 weeks and 16 weeks after planting

* - Statistically significant differences ($p < 0.05$) compared to control.

The level of carotenoids was increased by 26% in 16-week-old plants compared to the control in both variants without significant differences between the experimental samples (Figure 1D), while in 12-week-old plants the level of carotenoids was increased by 36% preplanting fertilization, and decreased by 32% with postplanting fertilization.

The antioxidant activity of kale (Figure 1E) was high due to the individual antioxidants it contained. The total antioxidant activity in the leaves of 12-week-old plants of both experimental variants was not statistically different from the control. In the 16-week-old kale plants, early biochar treatment increased total antioxidant activity by 24%, but late treatment had no effect on this index.

Lipid oxidation of the plasma membrane in the control samples was higher than the test samples at both growth points. After 12 weeks, the decrease in this indicator was more pronounced in the variant with the presowing treatment compared to the postplanting treatment (42% compared to 28%). However, at week 16, the rate of lipid peroxidation in both experimental samples were statistically similar, whereas the MDA had decreased by 54 % (Figure 1F).

Figure 1G shows that the protein content in the control plants and test variants was statistically the same at week 12. After 16 weeks, the amount of protein increased compared to the control by 56%, but only in the variant with biochar treatment done 1 month post planting.

The results of experiments on determining the content of soluble sugars in the leaves of plants fertilized with biochar immediately at planting and 1 month after planting are shown in Figure 1H. Plants fertilized with biochar immediately at planting were characterized by a significantly higher content of soluble sugars compared to the control, by 30% at week 12 and 29% at week 16. Plants fertilized with biochar 1 month after planting had 18% and 20% higher content of sugars compared to control after 12 and 16 weeks, respectively.

3.2. The determination of the content phenolic compounds by HPLC under the influence of biochar

Fourteen phenolic acids and flavonoids were identified in the studied plant samples (Figures 2–7).

At week 12, control plants had eight identified phenolic compounds (Figure 2), early-fertilized plants had 14 (Figure 3), and late-fertilized plants had six (Figure 4). Biochar stimulated the synthesis of phenolic compounds to a greater extent at week 12 of development. Six phenolic compounds were identified in control samples at week 16 (Figure 5), early-fertilized plants had 14 (Figure 6), and late-fertilized plants had 10 (Figure 7).

Thus, a greater variety of phenolic compounds is found in kale samples treated with biochar at the time of planting. All the test variants had more phenolic compounds present in the older plants. The early treatment with biochar is probably necessary for increasing the nutritional value of kale.

3.3. The determination of the effect biochar on the gene expression of HCT, F3'H, and CHS

As can be seen from our studies, biochar increased the content of phenolic compounds in kale, regardless of the time of application (Figures 1A and 1B). Figure 8 shows that the relative mRNA levels of the HCT, F3'H, and CHS genes were also altered by biochar treatment. Early biochar treatment stimulated an 80-fold increase in HCT gene expression, a 17-fold increase in F3'H gene expression, and an almost 22-fold increase in CHS gene expression. Late fertilizer treatment stimulated a roughly 22-fold increase in HCT gene transcripts, a 23-fold increase in F3'H gene transcripts, and a 16-fold increase in CHS gene transcripts in kale (Figure 8).

4. Discussion

In our research the concentration of phytochemicals such as phenolic compounds, flavonoids, vitamin C, carotenoids, sugars, and proteins was higher in 16-weeks-old plants than in 12-weeks-old plants. It was reported that the age of the plant significantly influenced its chemical composition more than the split-dose N treatment and frost action (Groenbaek et al., 2016), where it was proven that soluble sugars were higher in 13- and 17-week-old plants compared to 8-week-old plants, glucosinolates were higher in 8- to 17-week-old plants, and flavonoid glycosides concentration was higher in 13-week-old plants compared to 8- and 17-week-old plants (Groenbaek et al., 2016). In this examination, it was demonstrated that biochar (content element: C 18.64%, Corg 7.23%, N 1.88%, P 1.51%, K 1.77%) boosted the phenolic compound, flavonoid, carotenoid, and sugar content in kale irrespective of the biochar treatment time. A study revealed that treating plants with solutions and mixtures of mineral salts such as KKS, NPK, and NP increased several times the accumulation of polyphenolic compound-based antioxidants, shikimate, quinate, salicylate and tocopherol, compared to control plants grown both in fields and greenhouses (Osuji et al., 2017). The presence of potassium in biochar may have synergistically induced the variable rise in carotenoid, protein, and sugar content in curly kale (Dedei et al., 2021). In their research, Wang et al. (2013) explained that potassium helps in regulating the amounts of chlorophyll levels by preventing its decomposition. We discovered in our study that the application of N, P, and K in low concentrations enhanced the sugar content, especially in plants fertilized with biochar immediately at planting (Figure 1H). According to another study, soluble sugars in nitrogen, phosphorus, or potassium deficient plants were present in concentrations multiple

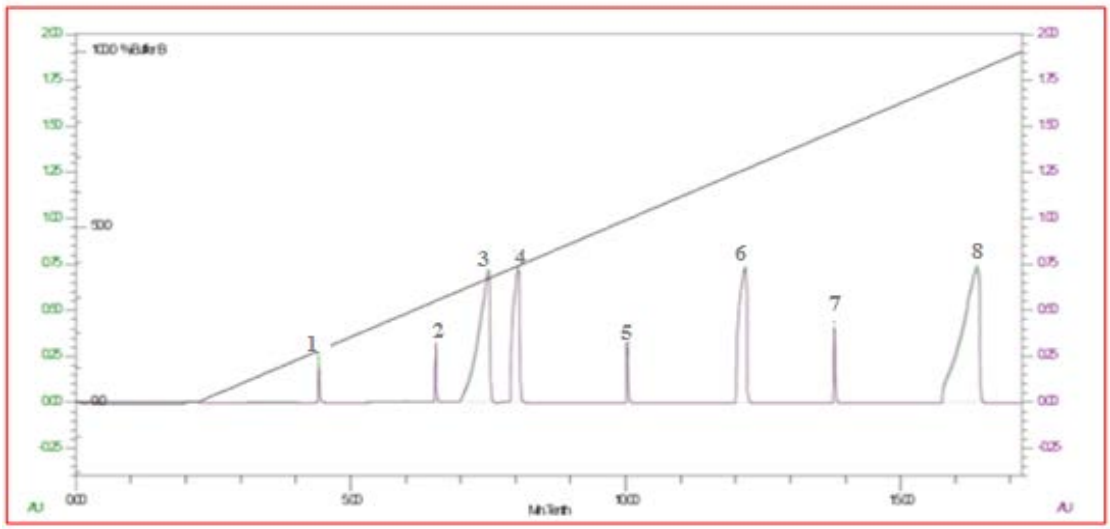

Figure 2. HPLC chromatogram of an alcohol extract of control kale plants (*Brassica oleracea* L. *var acephala*) (12w):1–isorhamnetin, 2–quercetin, 3–kaempferol, 4–sinapic acid, 5–ferulic acid, 6–benzoic acid, 7–caffeoylquinic acid, 8–cinnamic acid.

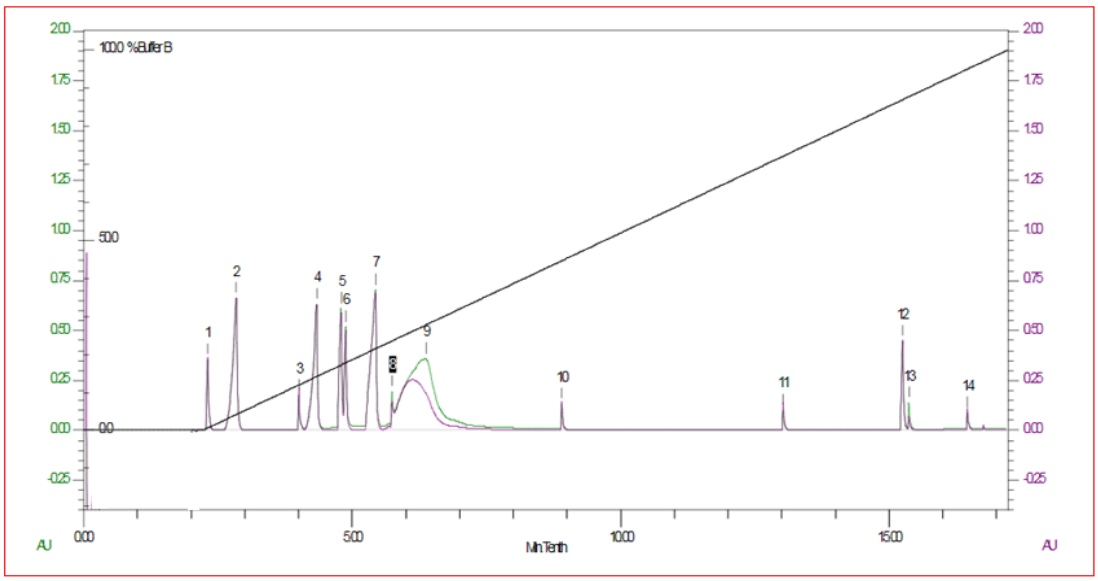

Figure 3. HPLC chromatogram of an alcohol extract of kale plants (*Brassica oleracea* L. var *acephala*) with biochar treatment at planting (12w): 1–gallic acid, 2–rutin, 4–isorhamnetin, 5–quercetin, 6–quercetin glycosides, 7–kaempferol, 9–sinapic acid, 10–sinapoyl-feruloyl-gentiobiose, 11–caffeoylquinic acid, 12–caffeic acid, 14–cinnamic acid.

times higher than in those with sufficient NPK (Sung et al., 2015). On the other hand, a significant decrease in the accumulation of soluble sugars in spinach has been reported in other studies (Okazaki et al., 2008). Our study shows that the content of protein increased compared to the control, but only in the variant with biochar treatment done 1 month post planting in 16-week-old plants (Figure 1G). The level of carotenoids also increased in 16-week-old plants compared to the control in both variants without significant differences between the experimental samples (Figure 1D), while in 12-week-old plants the level of carotenoids was higher in preplanting fertilization than in postplanting fertilization. Our results were aligned with the results of the study Yassin et al. (2021), in which they

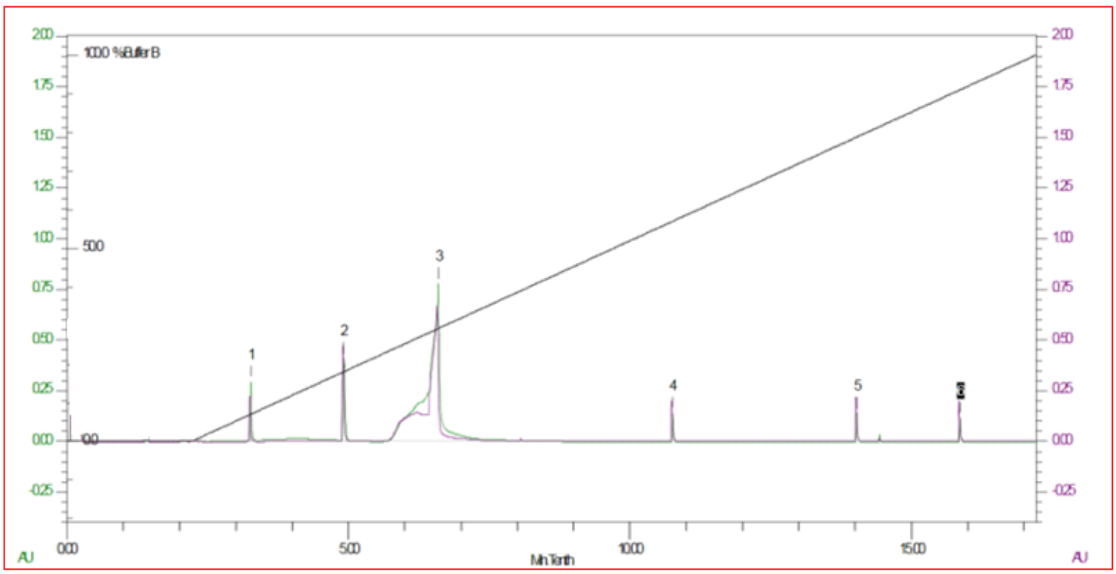

Figure 4. HPLC chromatogram of an alcohol extract of kale plants (*Brassica oleracea* L. var *acephala*) with biochar treatment 1 month after planting (12w): 1–rutin, 2–quercetin, 3–sinapic acid, 4– benzoic acid, 5–caffeoylquinic acid, 6–cinnamic acid.

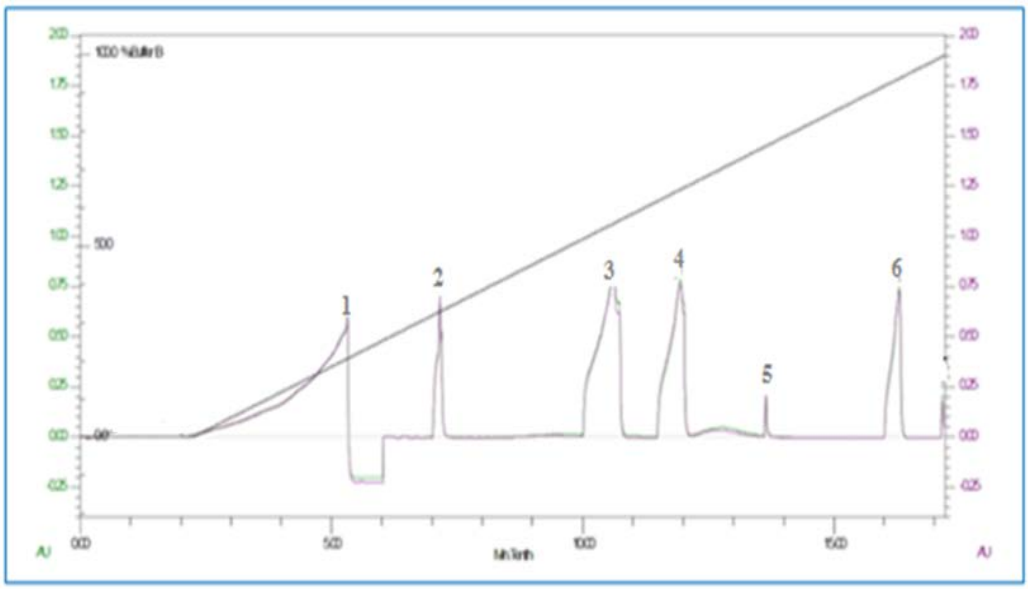

Figure 5. HPLC chromatogram of an alcohol extract of control kale plants (*Brassica oleracea* L. var *acephala*) (16w): 1–quercetin, 2–sinapic acid, 3–ferulic acid, 4–benzoic acid, 5–caffeoylquinic acid, 6–cinnamic acid.

proved that the addition of biochar to the soil increases the content of proteins and carotenoids in barley. After 12 weeks, the plants that were fertilized at planting had lower vitamin C content than the control plants by 45 %, while biochar applied 1 month after planting caused no significant changes in vitamin C content. This trend persisted at later stages of plant development (16 weeks) (Figure 1C). Previous work from Lata (2014) showed that N-fertility did not influence on ascorbate status of both kale cultivars (winterbor F1 and Redbor F1). Results from the work of Yassin et al. (2021) proved that adding biochar to the soil did not affect the content of vitamin C in barley.

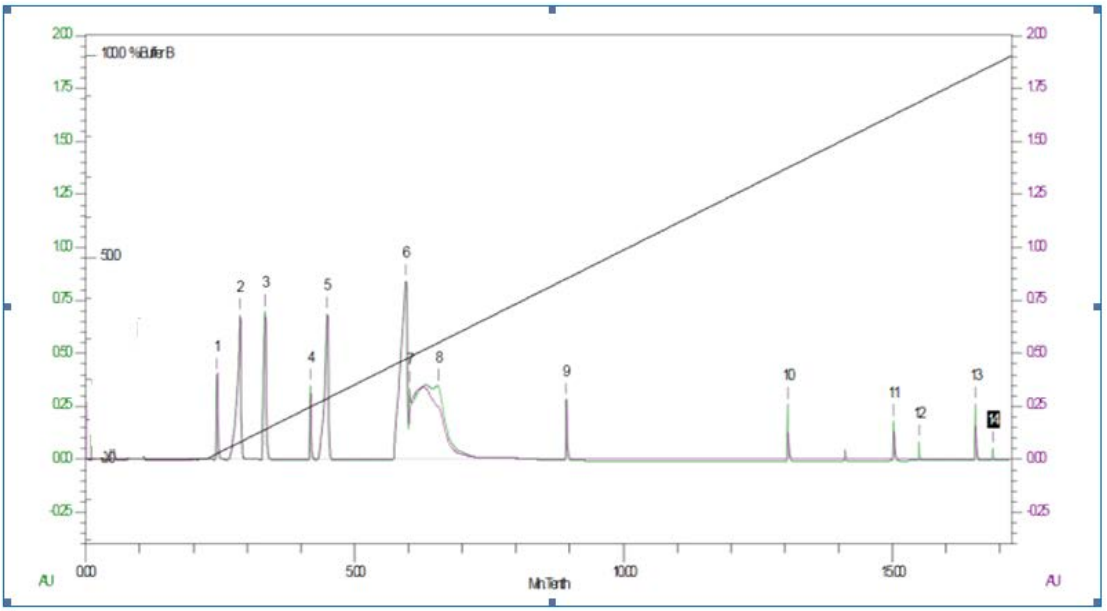

Figure 6. HPLC chromatogram of an alcohol extract of kale plants (*Brassica oleracea* L. var *acephala*) with biochar treatment at planting (16w): 1–gallic acid, 2–rutin, 3–isorhamnetin-3-O-rutinoside, 4–isorhamnetin, 5–quercetin, 6–kaempferol, 8–sinapic acid, 9–sinapoyl-feruloyl-gentiobiose, 10–caffeoylquinic acid, 11–caffeic acid, 13–cinnamic acid, 14–unknown.

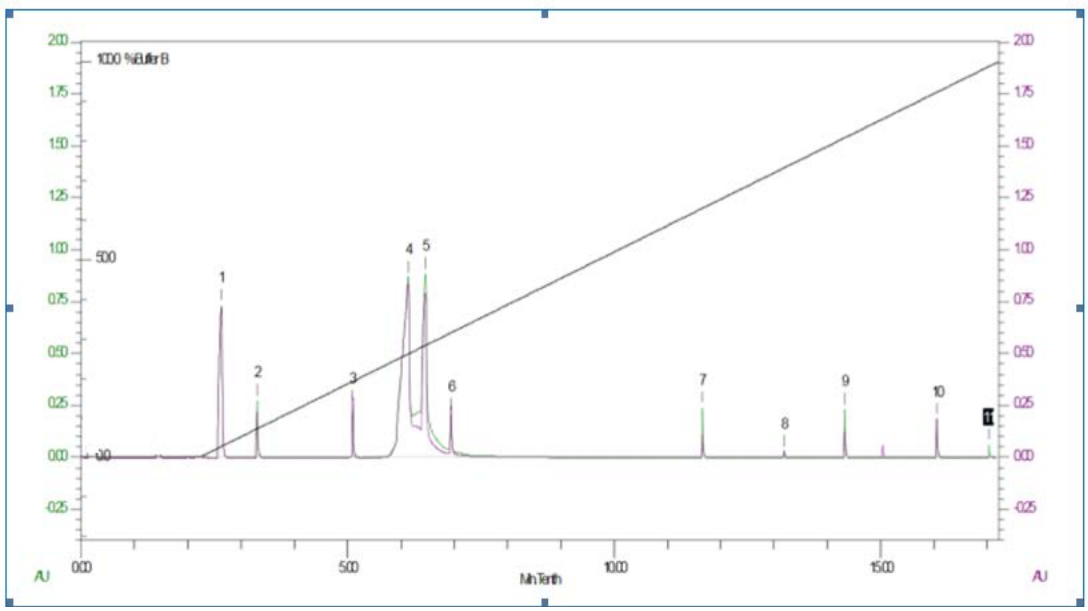

Figure 7. HPLC chromatogram of an alcohol extract of kale (*Brassica oleracea* L. var *acephala*) with biochar treatment 1 month after planting (16w): 1–rutin, 2–isorhamnetin, 3–quercetin, 4–kaempferol, 5–quercetin glycosides, 6–sinapic acid, 7–benzoic acid, 8–unknown, 9–caffeoylquinic acid, 10–cinnamic acid.

During our study, early biochar treatment increased total antioxidant activity in 16-week-old kale plants due to the increased content of enzymes and nonenzymes antioxidant under the influence of biochar. Our results were in agreement with the findings of Yassin et al. (2021), which concluded that biochar lowered MDA content and increased total antioxidant activity. Also, Ahmad et al. (2016) observed reduced levels of MDA in broad bean under the effect of potassium.

Phenolic compounds are very important classes of phytochemicals. The accumulation of phenolics, especially flavonoids, can be stimulated by a wide range of biotic and

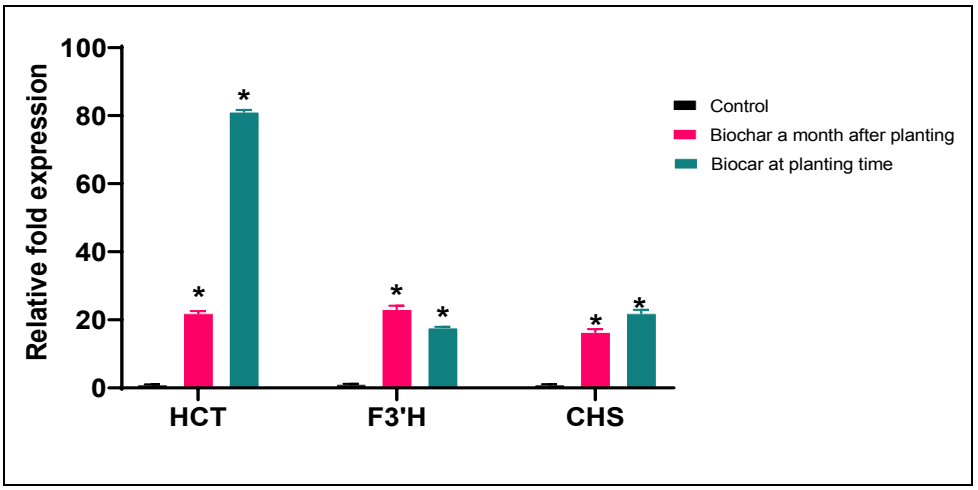

Figure 8. Number of hydroxycinnamoyl transferase (HCT), flavanone 3-hydroxylase (F3'H), and chalcone synthase (CHS) gene transcripts in cabbage (*Brassica oleracea* L. var acephala) treated with biochar * - Statistically significant differences ($p < 0.05$) compared to control.

abiotic factors, including nutrient availability (Groenbaek et al., 2016). Our experiment demonstrated that the content of phenolic compounds and flavonoids increased by a greater degree in the control plants that were 16 weeks old (Figures 1A and 1B). Regmi et al. (2023) reported that phenolic compounds and flavonoids concentrations varied widely by application different concentrations of biochar on *Viola cornuta* flowers.

The optimal relationship between N, carbon (C) and sulfur (S) is thought to exist in the interaction between primary and secondary metabolism, where protein synthesis, growth, and phytochemical biosynthesis have the ideal supply of their respective nutrients (Scheible et al., 2004). The optimal ratio depends on the specific phytochemical and corresponding pathway of its biosynthesis. Our results are consistent with the C-N balance theory (Stefanelli et al., 2010) which hypothesized that limited N may increase the synthesis of C-rich phytochemicals, such as phenolic compounds, flavonoids (and their derivatives), carotenoids, and sugars (Scheible et al., 2004), since the biochar we used contains more C than N.

In our study, the levels of phenolic compounds and flavonoids augmented under the influence of biochar at both growth stages studied, but their levels were higher in plants treated at planting (Figures 1A and 1B). This was also proven by the HPLC examination where biochar stimulated phenolic compound synthesis to a greater extent at 12 and 16 weeks of development (Figures 3 and 6). Fourteen phenolic compounds were identified in both experimental variants, as compared to the control samples (Figures 2 and 5). This can apparently be attributed to the ability of biochar to induce early reprogramming of gene expression for the synthesis of phenolic compounds. Many studies have shown that, in response to low nitrogen, there is a rapid reprogramming of gene expression that begins within the first hours after treatment (Fritz et al., 2006), while quantitative changes in metabolites are observed much later, after 2–7 days (Løvdal et al., 2010). This explains the increase in the content of soluble phenolic compounds and flavonoids after 16 weeks.

Numerous studies on trees, grasses, and annual crops have reported the effect of long-term and permanent nitrogen restriction on increased carbon-containing compound synthesis, including accumulation of phenols. The impacts of alternating periods of short-term nitrogen restriction (to stimulate phenol accumulation) and abundance (to support plant growth) have also been studied. The fate of elevated phenol concentrations after nitrogen replenishment has been studied in Arabidopsis (Olsen et al., 2009; Scheible et al., 2004) and tomatoes (Bénard et al., 2011). Although the levels of phenol biosynthesis gene transcripts were repressed within 1 day of nitrogen application, the decrease in tissue phenol concentration was slower and, apparently, metabolite-specific. Indeed, in Arabidopsis seedlings, the concentrations of rutin, cinnamic, and caffeic acids persisted for at least 24 h after nitrogen replenishment, while the concentration of ferulic acid decreased during the same period (Scheible et al., 2004). In tomatoes, the concentration of chlorogenic acid and rutin in the leaves of plants temporarily deprived of nitrogen was higher than in control plants within 5 days after nitrogen replenishment (Bénard et al., 2011). The increased content of phenols observed in our study can be explained by the fact that biochar activated the phenylpropanoid pathway and consequently the high

expression of HCT, F3'H, and CHS genes (Figure 8), and increased mRNA levels of the enzymes (chalcon synthase and dihydro flavonol reductase) involved in flavonoid synthesis (Kovácik et al., 2007). At week 12, 6 phenolic compounds were extracted from plants with late fertilization, and9 were extracted at week 16, where isorhamnetin, kaempferol, and quercetin glycosides appeared. The synthesis of phenolic compounds activated by biochar appears to be a prolonged process, and the effects of biochar reflect only after a certain period. Kale's flavonoids comprise mainly flavonol derivatives, which are reported to consist of mono to penta glycosylation of the aglycones quercetin, kaempferol, or isorhamnetin. Further, they are often acylated with hydroxycinnamic acids (Schmidt et al., 2010b). A previous work revealed that nonacylated flavonol glycosides occur as mono-, di-, tri- and tetraglycosides, whereas the monoacylated glycosides are present as di-, tri- and tetraglycosides, and the diacylated ones exist as tetra and pentaglycosides (Schmidt et al., 2010b). Contributors of the acylation are p-coumaric, caffeic, ferulic, hydroxyferulic, or sinapic acid (Schmidt et al., 2010b). We extracted isorhamnetin, quercetin, kaempferol, synapic, ferulic, benzoic, caffeoylquinic, and cinnamic acids in 12-week-old plants and quercetin, synapic, ferulic, benzoic, caffeoylquinic, and cinnamic acids in 16-week-old plants. Despite the increased content of phenolic compounds and flavonoids in the 16-week-old plants (Figures 1A and 1B), the diversity of phenolic compounds was shown to be lower than in the 12-week-old plants, where an absence of isorhamnetin and kaempferol was noticed in the 16-week-old plants (Figures 2 and 5).

5. Conclusion

The results of the current study concluded that early biochar treatment stimulated the synthesis of carotenoids, sugars, and phenolic compounds in the 16th week of plants in cabbage kale (*Brassica Oleracea* L. var acephala). The increased content of phenols could be explained by the increased expression of genes which involved in the accumulation of phenolic compounds such as HCT, CHS, and F3'H. The experiment also demonstrated that the antioxidant activity increased more in the 16th week of plants which had been fertilized with biochar at the time of sowing, and the degree of lipid peroxidation decreased after 12 and 16 weeks with the pre and postplanting treatments.

Similarly, it was observed that the main effect of biochar for the studied phytochemicals and nutrients was evident at week 16 for plants which were fertilized with biochar at the time of planting. These experiments convincingly show how biochar treatment of kale during planting is much more effective. Therefore, we recommend cultivating kale plants indoors and adding biochar at the time of planting at any time of the year in order to get a rich source of nutritional antioxidants.

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Conflicts of interest

The authors declare no conflicts of interest.

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