



Effects of nanocarbon solution treatment on the nutrients and glucosinolate metabolism in broccoli

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ABSTRACT

The effects of a nanocarbon solution on the nutrients, glucosinolate metabolism and glucoraphanin pathway in broccoli were investigated. Significant positive linear relationships were observed between the nanocarbon solution and total protein yield, although effects on the soluble sugars, vitamin C and dry matter production were not observed. All nanocarbon solutions significantly increased the glucoraphanin content ($p < 0.05$), and the 18.75 L·ha⁻¹ nanocarbon solution maximally increased the glucoraphanin content by 22.9%. However, these treatments also significantly reduced the contents of glucobrassicin, 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin and neoglucobrassicin. Further research demonstrated that the 18.75 L·ha⁻¹ nanocarbon solution significantly upregulated the *MAM1*, *IPMI2*, *CYP79F1*, *FMOgs-ox2*, *AOP2*, and *TGG1* expression levels, which directly resulted in the accumulation of glucoraphanin and glucoerucin. This study provides insights into the prospective nanotechnological approaches for developing efficient and environmentally friendly nanocarbon solution for use on crops.

1. Introduction

More than 130 glucosinolate compounds have been found in the crucifer family. Comparing to the other *Brassica* crops, broccoli has been found to be rich in glucoraphanin and glucobrassicin (Brown et al., 2015; Fahey, Zalcmann, & Talalay, 2001; Li et al., 2021). Currently, more studies have focused on broccoli and its sulforaphane extract in medical research. In recent decades, sulforaphane has been found to play a key role in anti-cancer and preventing chronic and cerebrovascular diseases (Kamal, Akter, Lin, & Nazzal, 2020). Sulforaphane is the major hydrolytic product of glucoraphanin especially rich in the seeds, seedlings, developmental buds, and florets of broccoli, in a neutral environment catalyzed by myrosinase (Li et al., 2014; Li et al., 2021).

It has been proved that glucosinolate synthesis in Cruciferae crops mainly depends on the genotypes and the other influencing factors, such as the environments and genotype × environment interaction. We could find that more recent studies have focused on storage methods beneficial for shelf life by detecting the nutrient composition in the postharvest period of broccoli. Broccoli is a good source of fiber, vitamin C and total

protein, and as well as containing vitamins, folic acid, and glucosinolate. Therefore, as an important nutrition factor, the glucosinolate has been widely quantitatively and qualitatively analyzed in broccoli. (J. Huang et al., 2021).

Recently, the development of nanotechnology has played an important role in current science and technology. A number of nanocarbon materials have been widely used in the kinds of aerospace fields and research, such as medical, aerospace, and high-speed railway. Since the discovery and applications of nano materials including carbon nanotubes, carbon nano-onions, and graphene, nano technologies and materials have attracted more attention due to their extraordinary characteristics beneficial for the human and the earth. Over the past ten years, the use of nanocarbon materials has primarily been investigated within animal science, and only a few studies have been reported on their use in plant science, primarily for rice, tobacco, and corn (Li et al., 2021; Wan, Wang, Bai, Wang, & Xu, 2020). The impact of nanocarbon materials on *Brassica* plants has scarcely been examined; however, this issue is important and calls for further in-depth research in the future.

In recent years, carbon dots, single- and multi-walled carbon

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nanotubes, graphene oxide and fullerenes, these carbon-based nanomaterials have been developed as new engineered nanomaterials (ENMs) (Lahiani, Chen, Irin, Puretzy, Green, & Khodakovskaya, 2015). Carbonaceous nanoscale particles have been proven to possess the potential to strengthen carbon assimilation and photosynthesis which might increase fungi and disease resistance in rice, and enhance plant growth and water conservation in saline environments (X. Wang, Liu, Chen, Han, & Yuan, 2014). To date, the effect of nanocarbon solutions (diameter < 6 nm) on soluble sugars, total proteins, vitamin C, dry matter, and glucosinolate in broccoli florets has not been reported. For the first time, this study seeks to utilize the possible benefits of carbon nanomaterials and their interactions with plants by irrigation with a nanocarbon solution at different concentrations during the growth period. Overall, we propose that the nanocarbon solution could affect the nutrients, glucosinolate metabolism and glucoraphanin pathway in broccoli, which would provide new insights for the development of efficient and environmentally friendly nanocarbon solution in the field and *Brassica* crops.

2. Materials and methods

2.1. Plant materials and treatments

Broccoli (*Brassica oleracea* var. *italica*) cultivar ‘Zhongqing 16’ (F₁ hybrid) was bred by the Institute of Vegetables and Flowers-Chinese Academy of Agricultural Sciences (IVF-CAAS). Broccoli plants with 4 true leaves were planted in a greenhouse (plastic shed) on a farm (40°15'N, 116°83'E) on April 9, 2020. Three treatments coupled with one control were set with three replications (n = 3), and every replication contained 126 plants (n = 126). At 20 days after planting, a total of 0.00 L·ha⁻¹ (T0), 3.75 L·ha⁻¹ (T1), 11.25 L·ha⁻¹ (T2) and 18.75 L·ha⁻¹ (T3) nanocarbon solution (0.6 %) with more than 95.5 % nanomaterials (diameter < 6 nm) was irrigated all at once by a drip irrigation system (Fig. 1). In addition, normal field management was carried out over the plant growth cycle. The nanocarbon solution was obtained from the Chinese Fertile Field and Ecofriendly Solution Limited Liability Company (Ltd) (Shandong). When the broccoli was harvested on May 25–31, broccoli florets (head) with diameters of 14 to 16 cm were randomly collected at the same time, and 5 samples (n = 5) were harvested in every replication. All samples were stored in a -80 °C

freezer until use. A number of fresh samples were pretreated to determine the solute sugar, vitamin C and total protein contents. The other collected samples were dried using a GRI 25–65 MP freezing-drying instrument, and the freeze-dried plant material was ground into a suitable powder with a coffee mill and stored at -40 °C for glucosinolate extraction.

2.2. Reagents

All chemicals and reagents were the highest purity. The internal standard glucotropaeolin was purchased from Sigma–Aldrich (St. Louis., MO, USA). HPLC-grade methanol and acetonitrile were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Deionized water was further purified by a Milli-Q Ultrapure water purification system (Millipore, Billerica, MA) and used in all experiments. C-18 solid-phase extraction (SPE) columns packed with 500 mg of sorbents were obtained from Grace (Deerfield, IL).

2.3. Measurement of soluble sugars, total proteins, vitamin C and dry matter

The soluble sugar content of the broccoli florets was extracted and quantified by a modified method. Approximately 0.10 g of ground fresh sample (homogenate) was extracted with 80 % (v/v) ethanol at 80 °C for 30 min and then centrifuged (Thermo Fisher Scientific Inc., USA) at 10000 × g for 10 min. The residue was extracted two more times using 80 % ethanol. The three supernatants were combined to an exact volume of 5 mL by adding 80 % ethanol. The soluble sugar contents were determined spectrophotometrically at a wavelength of A620 nm.

The total protein content was determined by Coomassie brilliant blue G-250 staining, and the nitrate content was determined by ion chromatography (GB 5009.5–2016). The content of vitamin C was determined by 2,6-dichloroindophenol titration (GB 5009.86–2016) (ZanOuyang et al., 2020). The content of dry matter was weighed and counted after pretreatment at 101–105 °C (GB 5009.3–2016).

2.4. Glucosinolate extraction and purification

The extraction of glucosinolate was based on hot methanol (Lou et al., 2008) with slight modifications. A total of 0.50 g dried samples

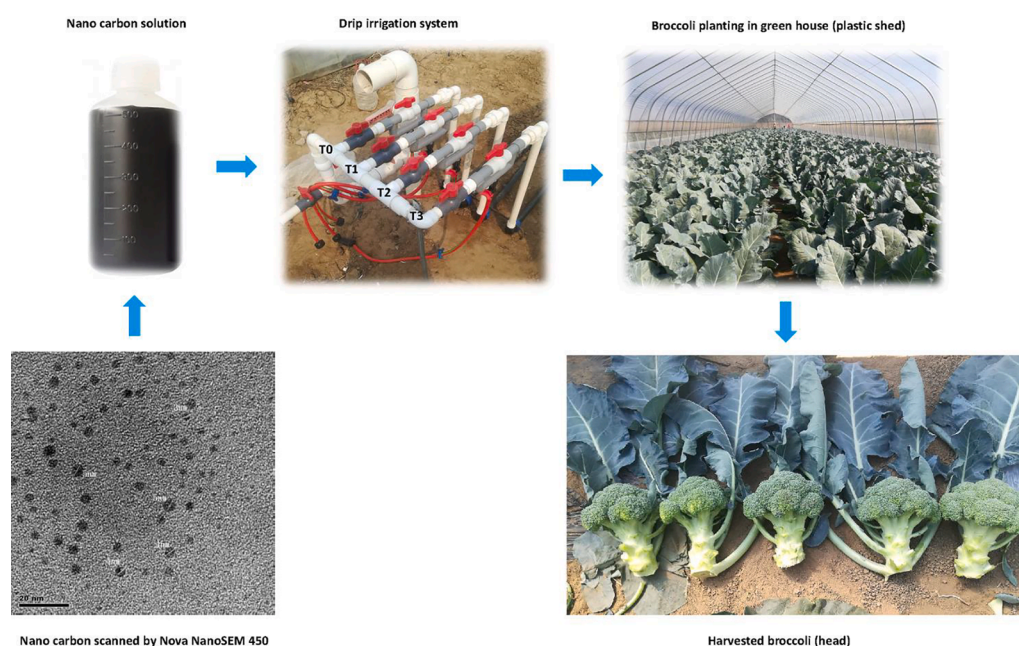


Fig. 1. Design of the nanocarbon solution effect on nutrients, glucosinolate metabolism and glucoraphanin pathway in broccoli.

were transferred to a 15 mL Greiner tube adding 5 mL of methanol (100 %) at 75 °C for 20 min. Meanwhile, 0.25 mL glucotropaeolin (3 mM) as the internal standard was added. Then, the mixture was centrifuged at 6500 rpm for 10 min, and the supernatant was collected and transferred to a new 15 mL Greiner tube. The pellet was reextracted twice with 70 % methanol (5 mL) at 75 °C, and the collected supernatant was mixed together.

The extracted supernatant of glucosinolates was purified on a Dry DEAE Sephadex A-25 (100 mg) anion exchange column (1.5 cm) (Bio-Rad Laboratories, Hercules, CA). A total of 2.0 mL of glucosinolate extract was added and washed twice with Millipore water (1.0 mL). Then, the column was washed twice with 1.0 mL of NaAC solution (0.02 M). Finally, 75 µL of sulfatase enzyme (25 mg/mL) (Dikma Technologies, CA, USA) was added and incubated overnight (16 h) at room temperature. Before the RP-HPLC analysis, the desulfated glucosinolates were eluted with Millipore water (3 × 0.5 mL) and filtered through a 0.22 µm filter.

2.5. HPLC analysis of glucosinolate

An Agilent HP 1100 Series HPLC system coupled to a diode array detection (HPLC-DAD) instrument (Agilent Technologies, Santa Clara, CA, USA) was applied for the quantitative analysis of glucosinolate compounds using an internal standard method, and benzylglucosinolate (glucotropaeolin) was selected as the internal standard. Analyte separation was conducted on a Nova-PakR C-18 column (3.9 × 150 mm, 5 µm) at 30 °C with a thermostated compartment. The two mobile phases consisted of 0.5 g·L⁻¹ ammonium acetate (A) and 100 % methanol (B) using the following gradient elution: 0.00–6.00 min, 100 % A; 6.01–21.00 min, 100 %–30 % A; 21.01–24.00 min, 30 %–0% A; 24.01–28.00 min, 0 % A (100 % B); 28.01–30.00 min, 0–100 % A, and 30.01–35.00 min, 100 % A. The injection volume of the sample was 20.0 µL, and the flow rate was 1.0 mL·min⁻¹. The UV detector at a wavelength of 229 nm was identified for desulfoglucosinolates, and wavelengths from 200 to 400 nm were scanned to identify glucosinolate compounds based on the retention time and the standard. The results are represented in units of µM·g⁻¹ ± SD DW (n = 3).

2.6. RNA extraction and real-time quantitative PCR (qRT-PCR) analysis

The total RNA of all samples was extracted and used for first-stand cDNA synthesis according to a protocol described previously by Li et al. (Li et al., 2019). The qRT-PCR was performed in a 25 µL reaction volume using SYBR Premix Ex Taq (TaKaRa, Dalian, China) with a 7900HT real-time PCR system (ABI, California, USA). The gene encoding actin-12 was used as an endogenous control according to our previous report (Li et al., 2014; Li et al., 2019). Specific primers for the targeted genes were designed with Premier 5.0 (Premier Biosoft, Canada) and were listed in Table S1.

2.7. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) using SPSS Statistics version 19.0 (SPSS, Inc., Chicago, IL, USA). One-way ANOVA and Tukey's multiple-range test were used to evaluate significant differences ($p < 0.05$). A two-tailed Pearson's correlation coefficient analysis and linear regression analysis were performed to evaluate the difference and relevance of the nanocarbon solutions, the nutritional values of the soluble sugar, protein, vitamin C and dry matter contents ($p < 0.05$), as well as the expression of the glucosinolate gene in broccoli. The statistical analyses and charts were produced using IBM SPSS software and GraphPad Prism 8.2.0 (GraphPad Software Inc., San Diego, CA, USA), respectively.

3. Results and discussion

3.1. Effects of nanocarbon solution on soluble sugars, total protein, vitamin C and dry matter

Plants are autotrophic and photosynthetic organisms that both consume and produce sugars simultaneously. Scientific research has shown that soluble sugars are highly sensitive to environmental stresses, which act on the supply of carbohydrates from source to sink organs (Wu et al., 2021). Soluble sugars act as metabolic resources and structural constituents of cells and as signals that regulate various processes associated with plant growth and development. Moreover, sugar signaling pathways interact with stress pathways to form a complex network that modulates metabolic plant responses, and soluble sugars also act directly as negative signals or as modulators of different plant processes, such as the plant hormones gibberellin (GA) and jasmonate (JA) (Zhang et al., 2021). Therefore, soluble sugars could play important roles in cell responses to stress-induced remote signals.

In this study, all treatments with the nanocarbon solution showed significantly increased vitamin C contents ($p < 0.05$) (Fig. 2a). Compared to the control, the treatments with 18.75 L·ha⁻¹, 11.25 L·ha⁻¹, and 3.75 L·ha⁻¹ nanocarbon solution increased soluble sugar contents by 9.4 %, 30.2 %, and 22.3 %, respectively. However, an obvious linear regression was not observed between all treatments and soluble sugars (Fig. 2b). In previous reports, single-walled carbon nanohorns (SWCNHs) and ZnO nanoparticles (ZnO NPs) were shown to have promoting effects on plant growth and salt tolerance in agricultural plants, such as rice, corn, soybean, and cucumber (S. Li et al., 2021). Moreover, foliar spraying with SWCNHs or ZnO NPs could increase the levels of soluble sugars and total protein, especially under salt stress (Martinez-Ballesta, Zapata, Chalbi, & Carvajal, 2016). According to previous reports, irrigation with the nanocarbon solution could efficiently increase the level of soluble sugar in broccoli.

Fig. 2 shows that the 3.75 L·ha⁻¹, 11.25 L·ha⁻¹, and 18.75 L·ha⁻¹ nanocarbon solutions also significantly increased the total protein and dry matter contents. Moreover, the 18.75 L·ha⁻¹ nanocarbon solution led to a 6.8 % increase in total protein and 3.9 % increase in dry matter in broccoli 'Zhongqing 16' and significantly increased the vitamin C contents compared to the other treatments at $p < 0.05$. A significant positive linear relationship was observed between the nanocarbon solution concentrations and total protein yield but not between the concentration and vitamin C and dry matter yield ($p < 0.05$). These findings provided new insights into obtaining the maximum total protein yield using a low-concentration nanocarbon solution by drip or sprinkler irrigation. Fresh broccoli is one of the most popular vegetables, and it presents high levels of protein, vitamin C, fiber, iron, and potassium. Dry matter usually accounts for 29 % of the dry weight of broccoli, which is high compared to most vegetables and healthy for humans. In general, one cup of chopped broccoli contains 2.5–3.2 g of total protein and 100 g fresh broccoli contains more than 2.8 g protein and approximately 34–36 calories. Therefore, broccoli is an excellent plant-based source of protein and a superfood for losing weight and belly fat (Armah et al., 2013).

Correlation analyses identify and evaluate the relationship between two variables. To better understand the relationship between the nanocarbon solution concentration and the broccoli nutrient content, a correlation analysis of the nanocarbon solution and individual nutrients was carried out in this study. Table 1 showed that a significant positive correlation occurred between the concentration of the nanocarbon solution and the total protein content in broccoli ($p < 0.01$), which was consistent with the linear regression analysis. Therefore, Fig. 2 indicated that the nanocarbon solution could significantly increase the contents of soluble sugars, total protein, vitamin C and dry matter. Meanwhile, a strong positive linear relationship appeared to occur for the nanocarbon solutions ranging from 3.75 L·ha⁻¹ to 18.75 L·ha⁻¹ and the total protein content in broccoli ($R^2 = 0.988$). Therefore, the use of a nanocarbon

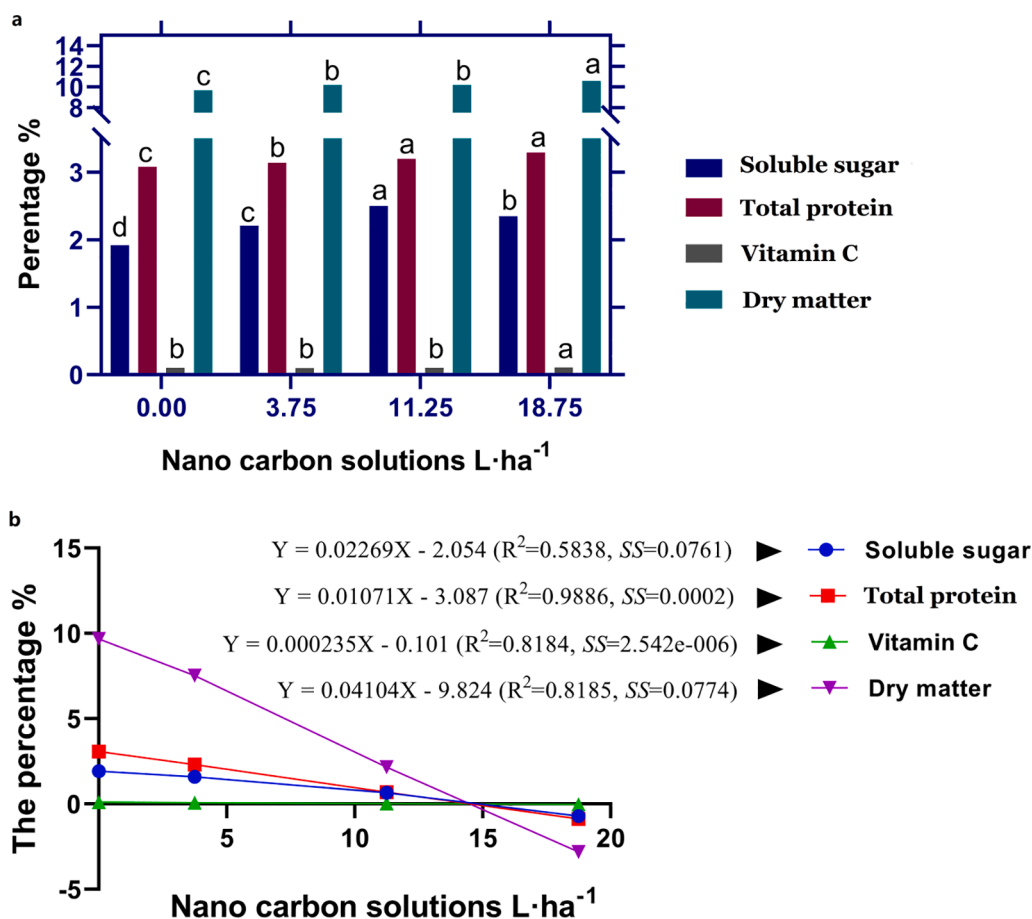


Fig. 2. Statistical analysis of the nano-carbon solution and nutrients in broccoli. The letters in the colored bars show significant differences in the nanocarbon solution that affected the contents of soluble sugars, total protein, vitamin C and dry matter based on one-way ANOVA at $p < 0.05$ (two-tailed) (a). The linear regression between the nanocarbon solution and nutrients (soluble sugars, total protein, vitamin C and dry matter) is shown in (b) at $p < 0.05$, and SS is short for the sum of squares.

Table 1
The coefficient of similarity analysis between the treatments and broccoli nutrition.

Pearson test		Concentrations	Soluble sugar	Total protein	Vitamin C	Dry matter
Concentrations	Coefficient	1	0.764	0.994**	0.905	0.905
	Significant		0.236	0.006	0.095	0.095
Soluble sugar	Coefficient	0.764	1	0.762	0.444	0.755
	Significant	0.236		0.238	0.556	0.245
Total protein	Coefficient	0.994**	0.762	1	0.878	0.944
	Significant	0.006	0.238		0.122	0.056
Vitamin C	Coefficient	0.905	0.444	0.878	1	0.704
	Significant	0.095	0.556	0.122		0.296
Dry matter	Coefficient	0.905	0.755	0.944	0.704	1
	Significant	0.095	0.245	0.056	0.296	

Note: ** represented the significant at $p < 0.01$ (two-tails), and * represented the significant at $p < 0.05$ (two-tails).

solution as a green fertilizer might provide new insights for improving crop production and nutrient yields and meeting the growing global demands for food, feed and fuel while practicing sustainable agriculture.

The present global population of 7.7 billion is projected to increase to nearly 9.8 billion by 2050, with the greatest increases occurring in developing regions, which will require at least a 50 % increase in the production of food and other agricultural products; moreover, a dietary transition toward the higher consumption of meat, fruits and vegetables relative to that of cereals will be observed from 2012 levels until the middle of the century (Kah, Tufenkji, & White, 2019). Agriculture needs to become more productive and diversified to address changes in climate and greater limitations on natural resources. Therefore, producing more food and nutrients using fewer resources and super varieties based on modern technologies, including nanomaterials and gene-editing systems, while preserving and enhancing the livelihoods of farmers is a global trend that presents challenges and opportunities (An et al., 2022).

3.2. Effects of nanocarbon solution on glucosinolates

In general, glucosinolates are prevalent throughout 15 botanical families of the order Capparales, including Brassicaceae, Capparaceae and Resedaceae, and more than 350 genera and 3000 species belonging to the Brassicaceae family contain glucosinolates, and they are the most representative glucosinolate-containing plants in the human diet (Prieto, López, & Simal-Gandara, 2019). The most commonly consumed edible plants from the Brassicaceae family include broccoli, cauliflower, cabbage, Brussels sprouts, Chinese cabbage, radish, turnip, wasabi, black mustard and so on (Mazumder, Dwivedi, & du Plessis, 2016). To date, more than 220 known glucosinolates harbor the same core structure, and three different groups of aliphatic glucosinolates (AGs), aromatic glucosinolates (ARGs), and indolic glucosinolates (IGs) can be summarized based on the origin of the core amino acid (Fahey et al., 2001). AGs are mainly derived from methionine but also from alanine,

leucine, isoleucine, and valine; ARGs are derived from both phenylalanine and tyrosine; and IGs are derived from tryptophan. The main glucosinolates in *Brassica oleracea* are 4C and 3C glucosinolates (progoitrin, gluconapin, glucoraphanin and sinigrin), while those in *Brassica rapa* are 4C and 5C glucosinolates (gluconapin and glucobrassicinapin). There are approximately 9–12 glucosinolates found in *Brassica oleracea* (Z. Li et al., 2021). Previous studies have mostly focused on broccoli because it is rich in glucoraphanin, and this compound degrades the product sulforaphane with strong anticancer and potent antioxidant activity (Kamal et al., 2020).

The major glucosinolate in *Brassica oleracea* vegetables is aliphatic glucosinolate, which has been proven widely in previous reports. We used HPLC to detect glucosinolate compounds in the florets of broccoli using an internal standard (glucotropaeolin) (Fig. 3). Among these 11 glucosinolates, 7 glucosinolate compounds, namely, glucoiberin, progoitrin, sinigrin, glucoraphanin, glucoalyssin, gluconapin, and glucoerucin, are aliphatic glucosinolates, while 4 glucosinolate compounds, namely, 4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin, are indolic glucosinolates. No aromatic glucosinolate was found in this study. Among the glucosinolate compounds, the highest contents were observed for glucobrassicin, which ranged from 5.07 to 5.61 $\mu\text{M}\cdot\text{g}^{-1}$ DW, thus accounting for 30.7 % of the total glucosinolates (16.38–17.75 $\mu\text{M}\cdot\text{g}^{-1}$ DW) on average. The second highest contents were observed for neoglucobrassicin, which ranged from 3.87 to 4.94 $\mu\text{M}\cdot\text{g}^{-1}$ DW, thus accounting for approximately 24.9 % of the total glucosinolates on average. The third highest contents were observed for glucoraphanin, which ranged from 3.57 to 4.39 $\mu\text{M}\cdot\text{g}^{-1}$

DW, thus accounting for approximately 23.9 % of the total glucosinolates on average. Meanwhile, the other 8 glucosinolate compounds had average contents of $<1.00 \mu\text{M}\cdot\text{g}^{-1}$ DW (0.08–0.97 $\mu\text{M}\cdot\text{g}^{-1}$ DW), and together, they accounted for approximately 20.5 % of the total glucosinolates on average. The glucosinolate compounds with the lowest contents were sinigrin and gluconapin, with average values of 0.08 $\mu\text{M}\cdot\text{g}^{-1}$ DW and 0.09 $\mu\text{M}\cdot\text{g}^{-1}$ DW, respectively. The average glucosinolate contents of glucoalyssin, glucoerucin, glucoiberin, progoitrin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin were 0.14 $\mu\text{M}\cdot\text{g}^{-1}$ DW, 0.37 $\mu\text{M}\cdot\text{g}^{-1}$ DW, 0.43 $\mu\text{M}\cdot\text{g}^{-1}$ DW, 0.59 $\mu\text{M}\cdot\text{g}^{-1}$ DW, 0.80 $\mu\text{M}\cdot\text{g}^{-1}$ DW, and 0.97 $\mu\text{M}\cdot\text{g}^{-1}$ DW, respectively. Therefore, the two indolic glucosinolates glucobrassicin and neoglucobrassicin and aliphatic glucosinolate glucoraphanin are major glucosinolate compounds in broccoli florets of ‘Zhongqing 16’, with most of the glucosinolate compounds belonging to the aliphatic group. However, these major glucosinolate compounds are significantly lost when yellowing occurs during broccoli storage (Casajús et al., 2021).

Fig. 3 showed that the nanocarbon solution could significantly increase the aliphatic glucosinolate yields of glucoraphanin and glucoerucin but obviously decrease the contents of indolic glucosinolates, including glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, and 4-hydroxyglucobrassicin. Clear divergence from the secondary modification pathway of aliphatic and indolic glucosinolate biosynthesis was observed (Fig. 4). Therefore, the nanocarbon solution might indirectly affect the aliphatic glucosinolate 3 carbon chain length (3C) content but directly increase the aliphatic glucosinolate 4 carbon chain length (4C) content and decrease the aliphatic glucosinolate 5 carbon

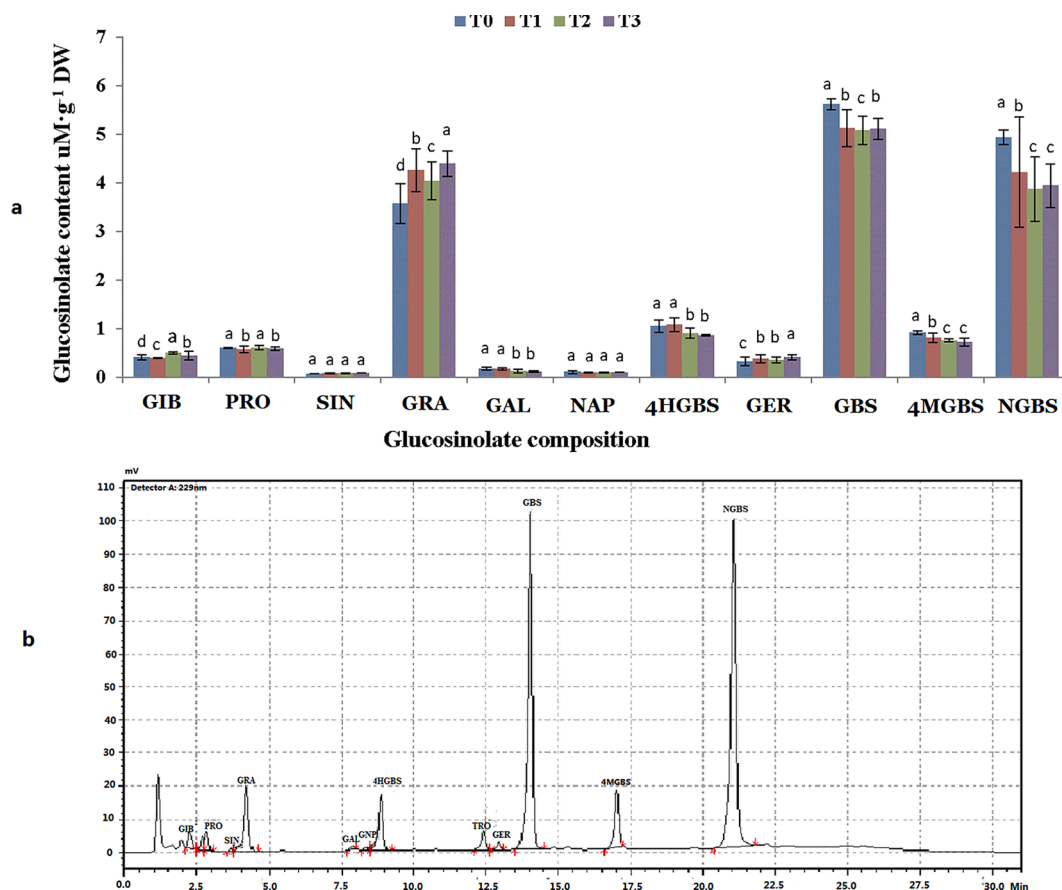


Fig. 3. Profile of the glucosinolate contents (a) and chromatogram (b) detected in broccoli florets by HPLC. (a) Effects of 3.75 L·ha⁻¹ (T1), 11.25 L·ha⁻¹ (T2), 18.75 L·ha⁻¹ (T3) nanocarbon solutions on glucosinolate content compared with the control (T0). Data are the mean \pm standard error from three biological replicate assays ($n = 3$). Different letters show significant differences ($p < 0.05$) for each sampling date among treatments. **GSL(s)**: glucosinolate(s); **GIB**: glucoiberin (3-methylsulfanylpropyl); **PRO**: progoitrin (2-R-hydroxy-3-butenyl); **SIN**: sinigrin (2-propenyl); **GRA**: glucoraphanin (4-methylsulfanylbutyl); **GAL**: glucoalyssin (5-methylsulfanyl); **GNA**: gluconapin (3-butenyl); **4HGBS**: 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl); **GER**: glucoerucin (4-methylthiobutyl); **GBS**: glucobrassicin (3-indolylmethyl); **4MGBS**: 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl); **NGBS**: neoglucobrassicin (1-methoxy-3-indolylmethyl).

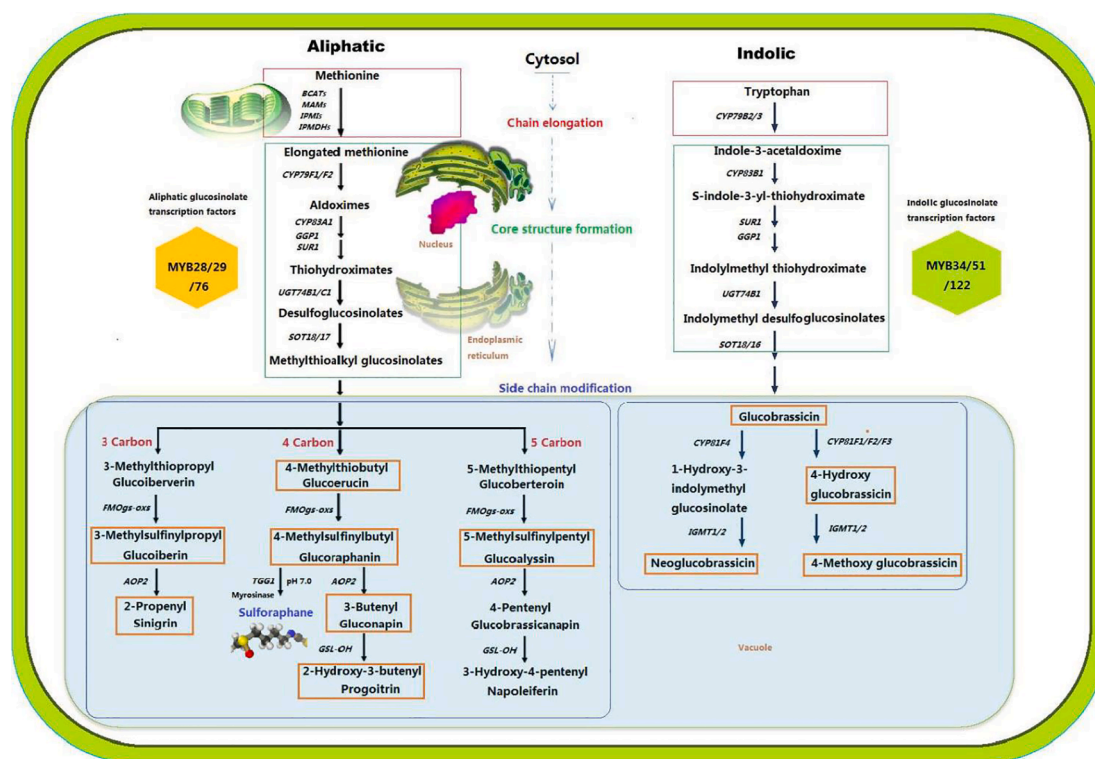


Fig. 4. Metabolic profile of the biosynthesis pathways for 3C, 4C and 5C aliphatic glucosinolates and indolic glucosinolates in *Brassica* plants. Black and blue arrows indicate the pathways for aliphatic and indolic glucosinolate biosynthesis, respectively. Glucosinolates in orange frames were detected in this study (broccoli florets). The rectangles in red, green, and blue represent the three steps of glucosinolate biosynthesis: chain elongation, core structure formation and side chain modification, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chain length (5C) yield. In this study, we also found that the nanocarbon solution could not increase the content of progoitrin (4C) downstream of the secondary modification pathway of aliphatic glucosinolate biosynthesis but might slightly increase the gluconapin (3C) content in the same branch (Fig. 4). These findings could provide new insights into efficient and environmentally friendly nanotechnological approaches for carbon solutions that can be used in the soil and in *Brassica* crops, which have rarely been reported in detail (Martínez-Ballesta et al., 2016).

Across the decades, studies on glucosinolate have increased dramatically, especially those focusing on its functional secondary metabolites, such as sulforaphanin (SFN), indole-3-carbinol (I3C) and 3-3'-diindolylmethane (DIM) (Huang, Lei, Wang, Sun, & Yin, 2021; Kim et al., 2019). Glucobrassicin represents the most widespread indolic glucosinolate present in cruciferous vegetables, and its breakdown products, such as I3C and DIM, and subsequent oligomerization products are well known as potential anticancer components of cruciferous vegetables (Kim et al., 2019). In our study, glucobrassicin was proven to be a major glucosinolate component in broccoli florets, which also suggested that broccoli is a natural plant with high nutrition for humans. Glucoraphanin, the precursor of SFN, has been intensively studied, and it's well known its second product SFN as a potential active molecule in immune boosting and anticancer activity, could provide powerful health benefits (Mrityunjaya, Pavithra, Neelam, Janhavi, Halami, & Ravindra, 2020). The breakdown products of neoglucobrassicin have been proved to inhibit the activation of Nrf2 target genes mediated by myrosinase-derived glucoraphanin hydrolysis products (Haack et al., 2010). In addition, sinigrin is usually present in the Brassicaceae family plants, which is used for its culinary and medicinal properties. In this research, further information about how to increase functional glucosinolates, such as glucoraphanin and glucobrassicin, using a nanocarbon solution was studied and provided in detail, and these data will be helpful for exploring the mechanism of glucosinolate biosynthesis.

3.3. Effects of nanocarbon solution on glucosinolate metabolism and pathway

Despite the great interest in glucosinolates associated with their possible roles in enhancing the defense mechanisms of plants under different biotic and abiotic stressors, the extensive variety of these metabolites increases the difficulty of decrypting the biosynthesis mechanism of each varietal (Prieto et al., 2019). *Arabidopsis thaliana* was initially used as the model plant to investigate the possible biosynthetic pathways of these compounds. So far, approximately 40 different glucosinolates have been detected in *Arabidopsis thaliana*, and most of these compounds are derivatives of methionine and tryptophan. A previous study showed that approximately 46 transcription factors and genes involved in glucosinolate biosynthesis were reported for *Arabidopsis thaliana* (Sønderby et al., 2010a). A total of 102 *Brassica rapa* glucosinolate biosynthetic candidate genes have been identified as homologs of 44 of the 52 known glucosinolate genes, and an additional 8 glucosinolate genes show no *Brassica rapa* orthologs (Wang et al., 2011). A total of 105 *Brassica oleracea* glucosinolate biosynthetic candidate genes and 3 AOP2 genes (with 2 nonfunctional genes due to the presence of premature stop codons) have also been found in the *Brassica oleracea* genome (02–12) (Liu et al., 2014). In contrast, all three AOP2 copies are functional in *Brassica rapa* while the AOP3 homolog is absent in *Brassica*, thus providing an explanation for why glucoraphanin is abundant in *Brassica oleracea* but lost in *Brassica rapa* (Sønderby et al., 2010; H. Wang et al., 2011).

In our study, to elucidate the effects of the nanocarbon solution on glucosinolate metabolism, especially on the glucoraphanin pathway, the expression profiles of the following 6 key genes related to glucosinolate metabolism were selected and detected in florets by RT-PCR: *MAM1* and *IPM12*, which are related to chain elongation; *CYP79F1*, which is related to core structure formation; and *FMOGs-ox2*, *AOP2* and *TGG1*, which are related to side chain modification (Fig. 4). Fig. 4 and Fig. 5a show that 4

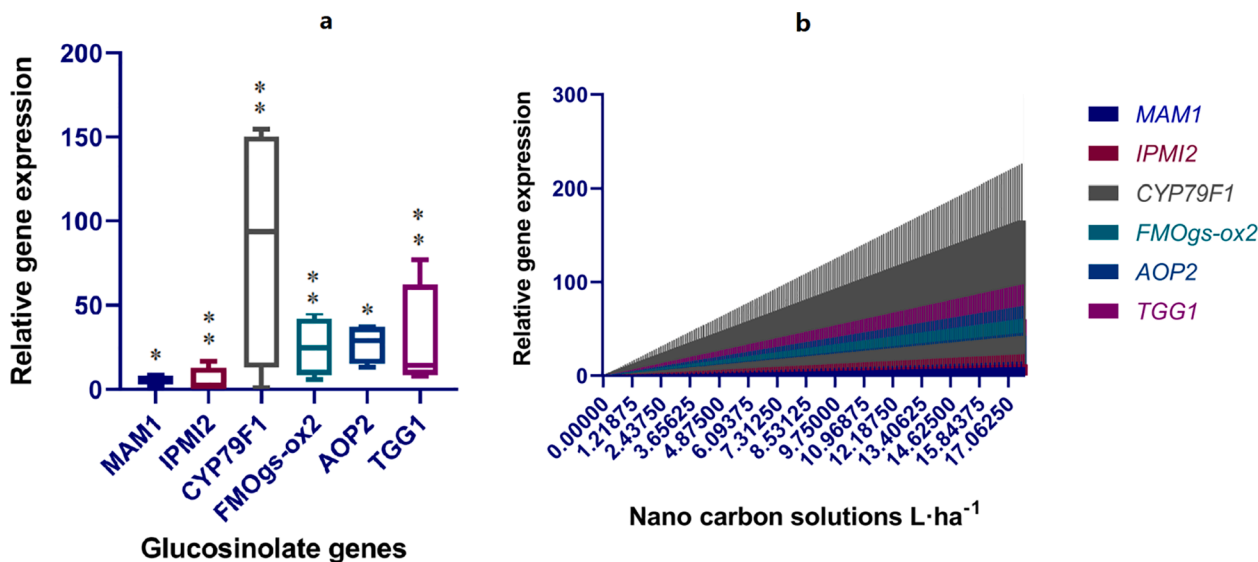


Fig. 5. Expression profile of the glucosinolate genes in broccoli florets. Significant differences were observed in the relative glucosinolate gene expression (*MAM1*, *IPMI2*, *CYP79F1*, *FMOgs-ox2*, *AOP2*, and *TGG1*) in florets treated with 0.00 (T0), 3.75 L·ha⁻¹ (T1), 11.25 L·ha⁻¹ (T2), 18.75 L·ha⁻¹ (T3) nanocarbon solution at $p < 0.05$ (*) and $p < 0.01$ (**). (a). (b) Linear regression analysis of the nanocarbon solution and glucosinolate gene expression levels in broccoli florets.

key upstream genes, *MAM1*, *IPMI2*, *CYP79F1* and *FMOgs-ox2*, were all obviously upregulated compared to the control (T0); moreover, the 18.75 L·ha⁻¹ nanocarbon solution (T3) strongly upregulated these genes, which are essential for glucoraphanin accumulation (Table S2). Similar effects also occurred for 2 downstream genes, *AOP2* and *TGG1*. These high levels of glucosinolate gene expression might provide evidence to confirm the positive action of nanocarbon solution on the accumulation of glucoraphanin and glucoerucin. The results of the linear regression analysis between glucosinolate gene expression and the nanocarbon solution (Fig. 5b) were similar to the above conclusion (Table S3).

Although *AOP2* gene expression was obviously upregulated after the nanocarbon solution in this study, the gluconapin content was not affected because the 2 bp deletion in the exon resulted in the malfunction of *AOP2* and glucoraphanin mainly accumulates in broccoli (Zhao et al., 2021). Meanwhile, the high level of 6 key glucosinolate genes did not seem to affect the contents of progoitrin and sinigrin, which were all at lower levels. The nanocarbon solution had a slight effect on increasing the glucoiberin content. At the same time, this result could not explain why there was a decrease in the glucoalysin content and significant decrease in all indolic glucosinolates, namely, glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, and 4-hydroxyglucobrassicin, which were further studied. The investigation of the *TGG1* expression level indicated that the treatment with 18.75 L·ha⁻¹ nanocarbon solution might greatly increase myrosinase synthesis and sulforaphane yield (Fig. 4), which would also provide new insights into enzyme engineering and utilization based on the development of nanocarbon.

4. Conclusion

The present study demonstrated for the first time that the applied nanocarbon solution not only significantly enhanced the yields of vitamin C and dry matter in broccoli but also had a great impact on the glucosinolate metabolism and biosynthesis pathways. According to an investigation of the effects of nanocarbon solution on nutrients, glucosinolate metabolism and glucoraphanin pathway in broccoli, significant positive linear relationships were observed between the nanocarbon solution concentrations and total protein yield. Moreover, among the 11 detected glucosinolates, glucoraphanin could be significantly increased by all nanocarbon solutions, with a maximal increase in glucoraphanin production (22.9 %) observed for the 18.75 L·ha⁻¹ nanocarbon solution.

The nanocarbon solution also significantly reduced the contents of 3 indolic glucosinolates, namely, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin, and nanocarbon solutions at greater than 11.25 L·ha⁻¹ also reduced the contents of 4-hydroxyglucobrassicin and the other indolic glucosinolates. Further research demonstrated that the 18.75 L·ha⁻¹ nanocarbon solution significantly upregulated the *MAM1*, *IPMI2*, *CYP79F1*, *FMOgs-ox2*, *AOP2*, and *TGG1* expression levels, which directly resulted in the accumulation of glucoraphanin and glucoerucin. This finding is supported by the linear regression analysis results between these gene expression levels and the nanocarbon solutions. This study provides new insights into prospective nanotechnological approaches for developing efficient and environmentally friendly carbon solutions for application in both the field and on crops.

CRedit authorship contribution statement

Zhansheng Li: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Guangmin Liu:** Methodology, Supervision, Validation, Visualization. **Hongju He:** Methodology, Supervision, Validation, Visualization. **Yumei Liu:** Resources, Conceptualization, Methodology, Project administration. **Fengqing Han:** Resources, Conceptualization, Methodology, Project administration. **Wei Liu:** Software, Investigation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2022.100429>.

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