Root-applied glycinebetaine controls nitrate content and edible quality of lettuce in continuous commercial hydroponic greenhouse production

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Lettuce (*Lactuca sativa* L.) may accumulate high amounts of nitrate $\left(NO_3^-\right)$ in hydroponic cultivation, such as in the Nutrient Film Technique (NFT) system. The NFT system is a commonly used hydroponic cultivation method in which a thin layer of nutrient solution flows continuously over the roots and is then recirculated. Since NO_3^- is known to be harmful to human health, lettuce producers need methods for controlling it and improving the quality of lettuce. Here, we investigated whether root-applied glycinebetaine (GB) can be used to control the NO_3^- content in commercial-scale greenhouse production. Three commercial-scale experiments were conducted with different GB application rates (0, 4, 7.5, and 10+10 mM GB). Glycinebetaine was applied to the nutrient solution, and lettuce was exposed to the GB treatment during the last 6 growing days before the harvest. Even though the root-applied GB slightly reduced the fresh weight of harvested lettuce and some minerals in lettuce dry matter, it significantly reduced the lettuce NO_3^- content (over 29%) and increased the contents of some amino acids in comparison to untreated plants. Thus, the edible quality of lettuce was improved. Furthermore, the tested GB application method proved to be functional on a commercial scale. Based on our study, adding GB to the nutrient solution can be considered a practical method for controlling lettuce NO_3^- content.

KEYWORDS

Lactuca sativa L., minerals, amino acids, NFT-cultivation, fertigation

Introduction

Lettuce (*Lactuca sativa* L.) contains essential vitamins, minerals, and health-promoting phytochemicals, such as carotenoids and polyphenols (Kimura and Rodriguez-Amaya, 2003; Kim et al., 2016). However, when lettuce is consumed in large quantities, the consumers' recommended acceptable daily intake (ADI) of NO_3^- may be exceeded (European Food Safety Authority, 2008). Since NO_3^- is classified as a harmful food additive in the EU, the European Commission has set a maximum NO_3^- content for fresh lettuce in

regulation 194/97 and requires the Member States to monitor the NO_3^- content of commercial lettuce. If the NO_3^- content in lettuce exceeds the set maximum the product will be rejected by traders. Thus, methods for controlling the NO_3^- content in lettuce leaves remain a major issue in commercial greenhouse production.

Commercial greenhouse-grown lettuce is commonly produced hydroponically in a continuous process, using the Nutrient Film Technique (NFT). In the NFT system, seedlings grow in small plugs of a growth substrate that are placed in narrow channels in which the nutrient solution flows continuously. Continuous production means a system in which the growth channels are set on a long table and continuously moved along the table until the plants reach commercial size. In this method, the plants have unlimited access to all the nutrients they need. Hence, lettuce may accumulate high amounts of NO_3^-. Lettuce takes NO_3^- up by roots from the nutrient solution. In the plant cell, the NO_3^- is reduced to nitrite and further assimilated into amino acids and proteins or stored in the cells divided into the vacuole and cytoplasm, as reviewed by Tischner (2000). Growing conditions, plant age, and fertilization affect the rate of NO_3^- assimilation (Blom-Zandstra, 1989; Santamaria et al., 1998; Zhang et al., 2018; Yi et al., 2020), and if NO_3^- uptake exceeds the rate of assimilation, NO_3^- accumulates in the vacuoles, resulting in high NO_3^- content in the leaves.

Many have expressed great interest in the means used to regulate the NO_3^- content of leafy vegetables. Especially the various fertilization strategies (Santamaria et al., 1998; Tabaglio et al., 2020). The effects of light intensity and spectrum on lettuce NO_3^- assimilation and content have also been of interest to researchers (Liu et al., 2016; Zhang et al., 2018; Yi et al., 2020). Reducing the NO_3^- concentration in the nutrient solution several days before harvest, replacing NO_3^- in part with ammonium (NH_4^+) at the end of the growing period, and removing the oldest leaves during the harvest, are methods previously tested in the commercial scale production of leafy vegetables (Santamaria et al., 1998, 2001). However, the use of NH_4^+ in the nutrient solution has been associated with stunted growth and reduced accumulation of fresh weight (FW) and leaf area (Santamaria et al., 1998; Hoque et al., 2008). The NH_4^+ may also damage leaves and roots (Hoque et al., 2008). Moreover, removing the oldest leaves during the harvest increases the workload and causes crop losses throughout the production chain. Light promotes photosynthesis and affects lettuce growth and metabolism. While growing lettuce indoors under a controlled climate and light-emitting-diode (LED) light, regulation of the light spectrum and photoperiod can be used to control lettuce quality (Viršilė et al., 2019). However, when growing lettuce in a greenhouse, especially in the Nordic countries, the proper light conditions may become unattainable, and therefore an additional precise method for regulating product quality is needed.

Osmolyte applications provide a novel approach to controlling lettuce NO_3^- content (Jokinen et al., 2022). Plants accumulate osmolytes (e.g., sugars, sugar alcohols, and NH_4^+ compounds) to maintain cell osmotic potential (Burns et al., 2010). The NO_3^- taken up by roots can replace these assimilates if photosynthesis is limited, which partly explains the increase in lettuce NO_3^- content under un-optimal growing conditions (Blom-Zandstra, 1989; Burns et al., 2010). Exogenous application of osmolytes, such as glucose and sucrose decreased NO_3^- uptake in pak choi (*Brassica rapa* var. *chinensis* L.) (Ma et al., 2018). Furthermore, Jokinen et al. (2022)

found that lettuce takes up root-applied glycinebetaine (GB), while GB treatment reduced NO_3^- content up to 40%, increased dry matter (DM) content, and affected the mineral -and amino acid profile in lettuce. In addition, Jokinen et al. (2022) reported that GB was stored in lettuce leaves in a concentration-dependent manner, while the leaf GB content was negatively correlated with leaf NO_3^- content.

Glycinebetaine (N, N′, N″ – trimethylglycine) is a quaternary ammonium compound that is endogenously accumulated in many higher plants (Wyn Jones and Storey, 1981; Rhodes and Hanson, 1993), and thus GB is a natural part of the human diet. However, lettuce does not accumulate endogenous GB (Jokinen et al., 2022). Glycinebetaine is considered a health-promoting compound in the human diet (Hayes et al., 2003; Craig, 2004). A dietary supplement GB reduced the plasma homocysteine concentration (Schwab et al., 2002), which is involved in a lower risk of cardiovascular diseases (Cho et al., 2006). Glycinebetaine also positively affects the gut microbiota (Koistinen et al., 2019).

Here we investigated the effect of GB on lettuce NO_3^- content and nutritional quality in commercial-scale hydroponic cultivation. We further focused the impact of GB on lettuce growth and adjusted the GB application to fit that of a commercial-scale continuous NFT-cultivation system. We hypothesized that lettuce accumulates GB from the nutrient solution reducing its growth while the edible quality (i.e., higher amino acid-and mineral content and lower NO_3^- content) of lettuce may be improved. The experiments were conducted in Finland in a commercial greenhouse during the winter and spring of 2020. This study consisted of three experiments, using the same greenhouse facilities and experimental design.

Materials and methods

Cultivation

Lettuce (cv. 'Danstar' or 'Frillice') seeds were sown in plastic pots (Ø 5 cm 0,08 L, PR30, VEFI EUROPE, (Skierniewice, Poland) VEFI Drammen, Norway) filled with fine peat (pH 6.0, electrical conductivity (EC) 0.21 S/m, Kekkilä Professional, VHM 620 R8060 + iron (Fe), Kekkilä Oy, Vantaa, Finland) at a rate of one seed per pot. The seeds were germinated in a climate-controlled dark room at 19°C for 1 day and transferred into a seedling line in a greenhouse under automatically controlled (Priva Connex; De Lier, Netherlands) climatic conditions. The day/night temperature was 20–24°C/17–18°C, and the relative humidity (RH %) fluctuated between 48 and 77%. In the first two experiments, conducted in January and February (2020), artificial high-pressure sodium (HPS) lights (average intensity 160 μmol m^{-2} s^{-1}) were used, and the day/night light period was set to 20/4 h. Artificial lights were not used in the third experiment conducted in May 2020, because there was already enough sunlight available, and the day length in Finland was over 16 h.

For seedlings over the first 15 days, the nutrient solution was prepared by mixing a 2.5% stock solution of Professional Superex Farm Edition (Nitrogen-Phosphorus-Potassium (N-P-K) 10–3-30, Mg 1.7, Kekkilä Professional, Kekkilä Oy, Vantaa, Finland) and 2.5% stock solution of calcium nitrate (Ca (NO$_3$)$_2$15.5–26.3, Van Iperen, International B.V.,Westmaas, The Netherlands). The stock solutions were mixed in a 1:1 ratio and automatically added to the irrigation

water according to the demand for EC, which was set to 0.11 S/m. The seedlings were watered with an irrigation ramp and spray nozzles. After the 15-day growing period in the seedling line, well-developed seedlings were transplanted to the NFT-channel tables for channel cultivation. The channel tables were 100 m long and 4 m wide. Each channel had 32 pots and the plant density was 25.6 plants m^{-2}. The seedlings were set in and harvested at the opposite ends of the channel table at the rate of 10 channels per day, hence the channels were moved forward along the table once per day. In general, the growing period from seeding to harvest fluctuated between 40 and 43 days.

Two fertigation stock solutions were manually prepared in separate tanks for channel cultivation. Solution A contained 100 kg 1,000 L^{-1} Kekkilä Professional Superex Farm Edition, and solution B 25 kg 1,000 L^{-1} Ca (NO$_3$). Stock solutions A and B were automatically added in equal amounts to the circulating nutrient solution reaching the EC demanded. The automatically regulated irrigation term took place after every 45 min. The EC of the circulating nutrient solution was set to 0.21 S/m in the first two experiments, and 0.18 S/m in the third experiment. The concentration of nitrate nitrogen (NO$_3$ –N) in the nutrient solution fluctuated between 180 and 200 mg L^{-1}. After every irrigation pulse, the returning water was filtered through sand (3–5 mm) and rock wool biofilters and led to a basin; the basin water was re-used. During the irrigation pulse, the greenhouse automatic (Vialux Hd/UV system, Priva, De Lier, Netherlands) adjusted the EC in the nutrient solution by adding fertilizer stock solutions or pure water disinfected with an ultraviolet (UV) filter.

Experimental design

The experimental area consisted of two adjacent NFT-channel tables in the middle of the block greenhouse. Table A was set up as the control table and table B was set up for GB treatment. Table A was connected to the automatic irrigation system generally used in the greenhouse. The channel table used for GB treatment was split into two water recycling sections (Figure 1). The first section was connected to the greenhouse's general irrigation system, and the plants were grown in it for approx. 20 days. Thereafter, the plants reached the treatment area where they were grown for 6 days before the harvest. The GB-treatment section consisted of 144 channels totaling 4,608 plants. A water tank (3,000 L) containing nutrient solution was connected to the GB-treatment section for automatic irrigation (Siemens Logo 8, Siemens AG, Munich, Germany), and the irrigation pulse was set at 45 min intervals corresponding to the irrigation interval used for the control plants.

At the beginning of the experiments, the water tank (3,000 L) was filled with the EC-adjusted nutrient solution. In the first two experiments, the EC was set to 0.21 S/m, and in the third experiment to 0.18 S/m. Glycinebetaine (Nutristim pure, Finnfeeds Finland Oy, Naantali, Finland) was applied in the tank by first dissolving it in small batches in 10 L of nutrient solution. The GB-containing nutrient solution was then mixed with an underwater pump (Grundfos Unilit KP 250, Grundfos Holding A/S, Bjerringbro, Denmark) for 1 h before it was led to the channels. Water consumption and the EC of the tank solution were monitored daily (Senmatic EC Mesur hand instrument, Senmatic A/S Industrivej, Søndersø, Denmark). The fertilizer stock solutions were manually added to the tank to maintain EC demand. In the third experiment,

the GB application was repeated 4 days after the first application, for which that the tank was refilled with fresh nutrient solution and GB was applied as described above.

Glycinebetaine treatment

In the first two experiments, the GB was applied once in the nutrient solution followed by an experimental period of 6 days. The GB concentration was set to 4 mM in the first experiment, and 7.5 mM in the second. In the third experiment, two GB applications (double-application) were used: the first dose of GB resulting in a 10 mM GB concentration in the solution was applied at the beginning of the experiment, and a second 10 mM dose of GB was applied 4 days later to continuously maintain the GB available for the plants (Figure 2). During the second GB application, the tank was refilled with fresh nutrient solution. The third experiment lasted 14 days. Thus, the channels were moved daily for the harvest, and the GB concentration in the nutrient solution was reduced over time, plants arriving at the treatment area were exposed to GB at different concentrations and times.

Plant sampling

In the first two experiments, the sampling was conducted daily over the 0–5 days after GB application (0–5 DAA). In the third experiment, the sampling was conducted at 0, 3, 5, 7, 10, 12, and 14 DAA. The plants were harvested at 40–43 days after seeding (DAS) when they had reached 100–200 g FW. At each sampling time at 10:00 am, one plant from the middle of each of the 10 channels was collected totaling 10 plants from the GB growing table and 10 plants from the control table. The plants were cut on the surface of the peat, and the lettuce head was weighed, and frozen for at least 2 s at −20°C. The nutrient solution was sampled simultaneously with plant samples from both lines and frozen for 24 h at −20°C.

Nitrate content in lettuce leaves

The NO$_3^-$ content in the lettuce leaves was measured with a Horiba LAQUA Twin 741 crops meter (Horiba Advanced Techno Co. Ltd., Kyoto, Japan). In brief, six frozen samples of each of the sampling times were allowed to thaw at 5°C overnight followed by at least 2 h at room temperature. The thawed samples were squeezed with a potato press. The NO$_3^-$ content was measured four times from each liquid sample and the average of measurements was multiplied by a correction factor of 0.77 according to Näkkilä et al. (2015). The NO$_3^-$ meter was calibrated after every fourth measurement using a two-point calibration procedure with Y042 300 parts per million (ppm) and Y041 5,000 ppm Nitrate Ion Standard Solutions (Horiba).

Chemical analyses

All the chemical analyses conducted in this study were earlier used and described in Jokinen et al. (2022). In brief, the remaining frozen plants from the third experiment (4 GB-treated and untreated plants in each of the sampling times) were freeze-dried, weighed,
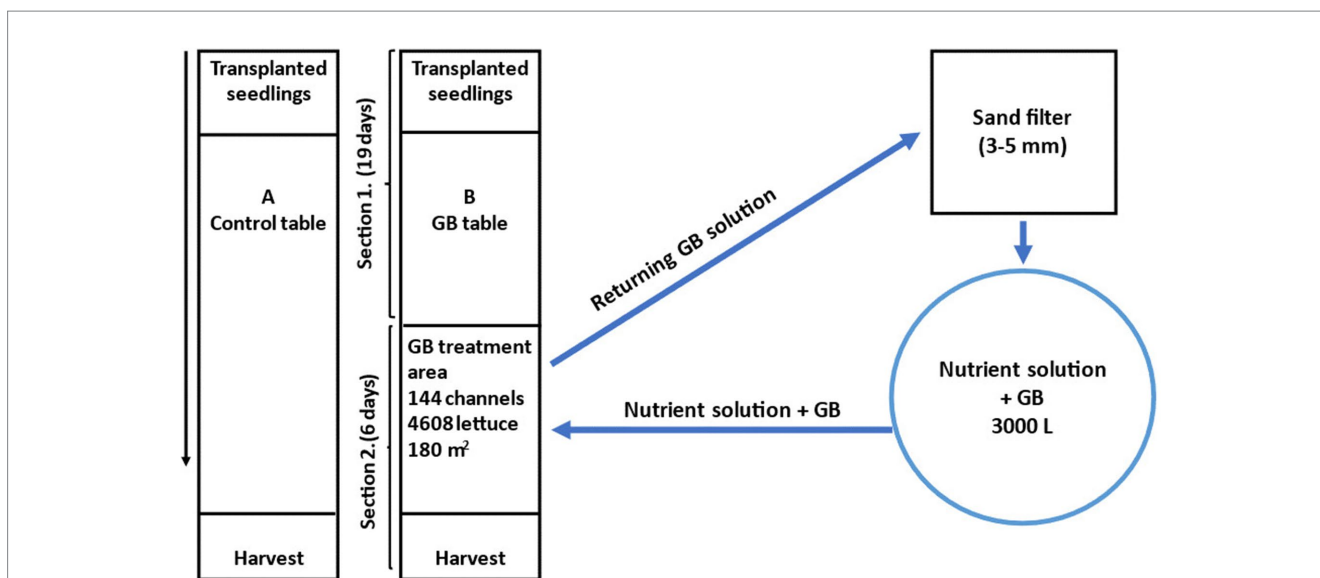
FIGURE 1
Floor plan of the glycinebetaine (GB) experiment. Lettuce (*Lactuca sativa* L.) seedlings were transplanted in NFT-channel cultivation at 15 DAS and let grow until harvest size (> 100g) was reached. The channels were moved along the tables continuously; every day 10 channels of lettuce were harvested, and new seedlings were put in. The GB solution was let into the treatment area through a separate water recycling system. The plants reached the treatment area approx. 34 DAS and were harvested at 40−43 DAS. DAS=days after seeding. NFT=Nutrient Film Technique.
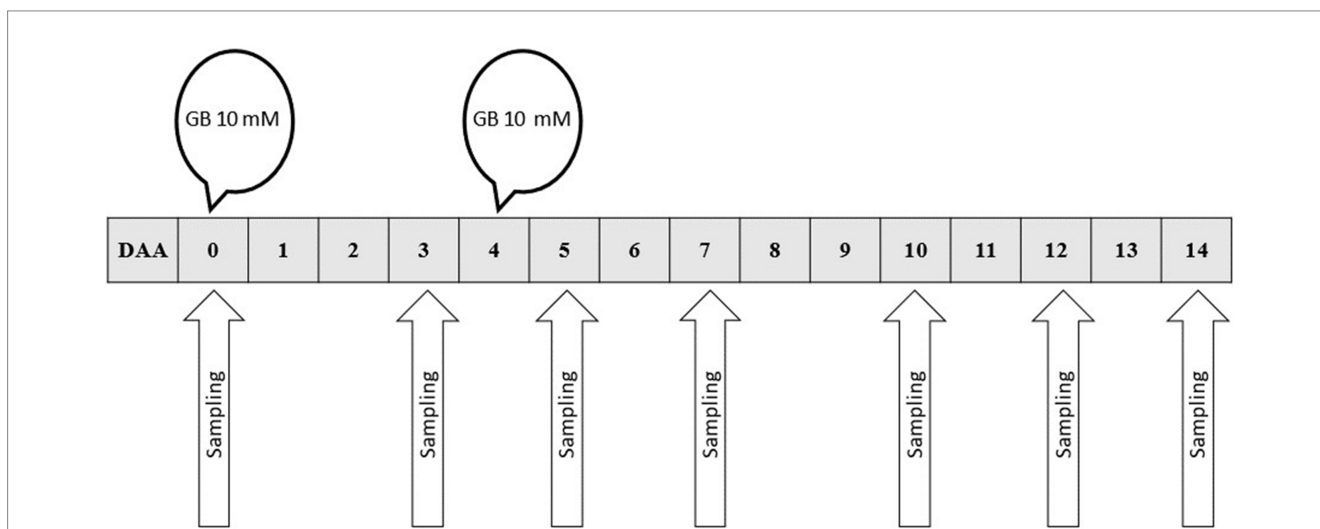

FIGURE 2
Experimental protocol with GB double-applications (10+10mM). The plants were sampled at different times (DAA) and were grown in GB-containing system as follows; 0, no GB; 3, one GB dose 3days before harvest (DBH); 5, GB dose five and one DBH; 7, GB dose seven and three DBH; 10, GB dose 10 and 6 DBH; 12, GB dose 12 and 8 DBH; 14, GB dose 14 and 10 DBH. DAA=days after application. GB=glycinebetaine.

ground (1 mm sieve, Retsch Grindomix GM 200; Retsch GmbH, Haan, Germany), and stored at −20°C until further analysis. The ground dry samples were used for chemical analyses. The GB content was analyzed with ultrahigh-performance liquid chromatography (UHPLC) (Water Acquity Ultra Performance LC; Waters Corp. Singapore) equipped with a light-scattering detector using a Cortecs hydrophilic interaction liquid chromatography (HILIC) column (2.1 × 150 mm, 2.7 µm; Waters). Glycinebetaine concentration in the fertigation solutions was analyzed using the same method. For the analyses, samples of nutrient solution were diluted 1:5 with acetonitrile (acetonitrile: water (9:1 v/v)). The total nitrogen (N) and carbon (C)

contents were analyzed with Duma's combustion method (CN 828, Leco Corp., St. Joseph, MN), and the other mineral contents (Ca, Fe, K, magnesium Mg, manganese Mn, sodium Na, P, sulfur S, and zink Zn) with inductively coupled plasma-optical emission spectrometry (ICP-OES, iCAP 6,200; Thermo Fisher Scientific, Cambridge, United Kingdom). The amino acids were analyzed with an ACQUITY UPLC system (Waters Milford, MA, United States) consisting of an Acquity photodiode array (PDA) optical detection system. A Waters BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm; Waters Milford USA) was used. Empower 2 (Waters, USA) software was used for system control and data acquisition.

Data analysis

Data on the growth and contents of NO_3^-, GB, minerals, and amino acids were analyzed with the IBM SPSS data editor (Version 26, Armonk, NY, USA). A two-way analysis of variance (ANOVA) was performed to show the effects of the treatment (Trmt), the effect of time after the first GB application (DAA), and their interaction. Significant differences were considered when the p-values were < 0.05. If a significant difference within the DAA x Trmt interaction was observed, the means in every sampling time were compared, using Tukey's multiple range test.

Results

Glycinebetaine in the fertigation solution and in leaves

The GB was detectable in the nutrient solution over 3 DAA after 4 mM application (Figure 3A), and 4 DAA after 7.5 mM application

(Figure 3B). In the third experiment, the GB concentration detected in the fertigation solution was 4 mM after the first application, even though the targeted concentration was 10 mM (Figure 3C). However, the GB concentration was below the detection limit 3 DAA. After the second application, the GB concentration in the fertigation solution reached a peak value of 6.6 mM at 7 DAA and was again below the detection limit at 11 DAA until the end of the experiment. Samples of the fertigation solution were not collected at 2, 8, and 10 DAA.

In the third experiment, the lettuce accumulated root-applied GB in its leaves. Following the first 10 mM GB application, the leaf GB content was 106 μg g^{-1} DM at 3 DAA (Figure 3D). The GB content in the leaves increased further after the second GB application attaining maximal value of 128 μg g^{-1} DM at 5 DAA.

Nitrate content

The plant NO_3^- content varied over time in the treated and untreated plants. Following the 4 mM GB application, no clear response was observed (Figure 4A). The NO_3^- content in lettuce
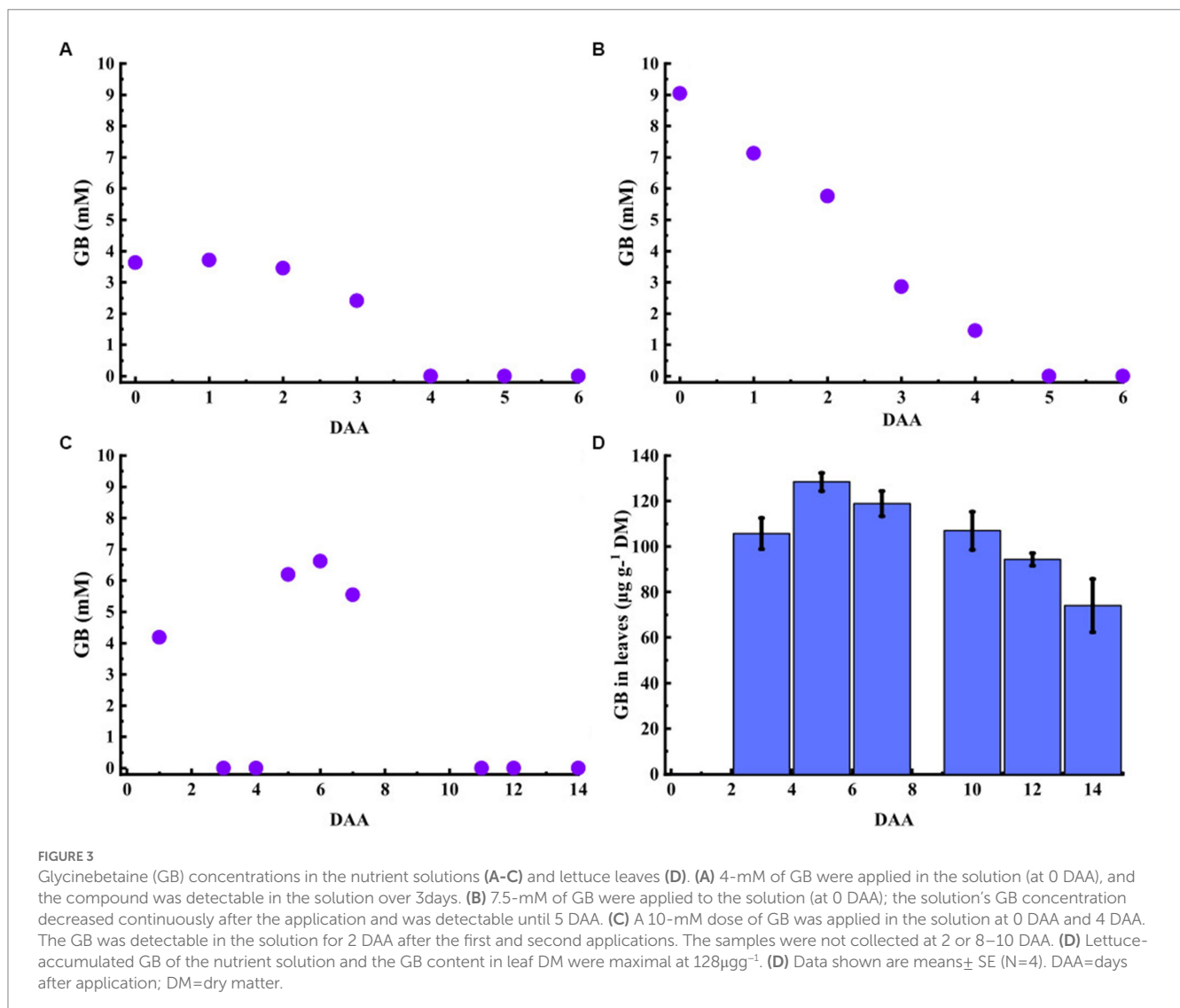
FIGURE 3
Glycinebetaine (GB) concentrations in the nutrient solutions **(A-C)** and lettuce leaves **(D)**. **(A)** 4-mM of GB were applied in the solution (at 0 DAA), and the compound was detectable in the solution over 3days. **(B)** 7.5-mM of GB were applied to the solution (at 0 DAA); the solution's GB concentration decreased continuously after the application and was detectable until 5 DAA. **(C)** A 10-mM dose of GB was applied in the solution at 0 DAA and 4 DAA. The GB was detectable in the solution for 2 DAA after the first and second applications. The samples were not collected at 2 or 8−10 DAA. **(D)** Lettuce-accumulated GB of the nutrient solution and the GB content in leaf DM were maximal at 128μgg^{-1}. **(D)** Data shown are means± SE (N=4). DAA=days after application; DM=dry matter.

FIGURE 4
Effect of glycinebetaine (GB) on lettuce (cv. **A,B** ='Danstar', **C** ='Frillice') nitrate content. **(A)** The GB (4mM) was applied into the nutrient solution at 0 DAA. **(B)** The GB (7.5mM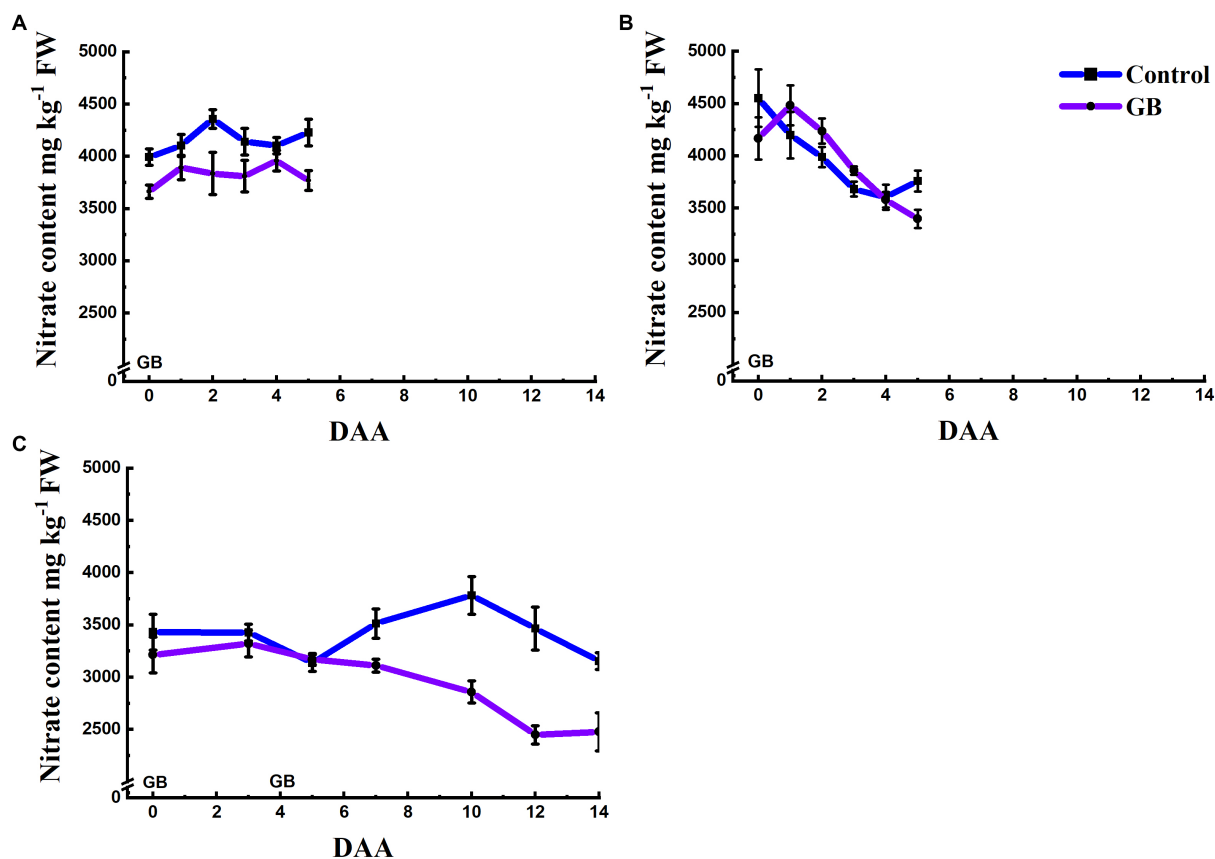) was applied into the nutrient solution at 0 DAA. **(C)** The first dose of GB (10mM) was applied into the nutrient solution at 0 DAA, and the second dose of GB (10mM) was applied at 4 DAA. Each plant entered the treatment area 6 days before harvest. Data shown are means $\pm$ SE (**A,B** : N=10; **C**: N=6). FW=fresh weight; DAA=days after application.

leaves was reduced, following the 7.5 mM GB application when the plants were exposed to GB over 3 days (Figure 4B). In the double-application GB treatment (10 + 10 mM GB), the NO_3^- content of the lettuce leaves decreased, and a significant interaction ($p < 0.05$) between DAA and Trmt was observed. The NO_3^- content in the GB-treated plants remained lower in comparison to the untreated plants from 6 DAA onwards (Figure 4C). The difference in the NO_3^- content between the GB-treated and untreated plants peaked at 12 DAA when the NO_3^- content in the GB-treated plants was 2,447 mg kg^{-1} FW and in untreated plants 3,465 mg kg^{-1} FW. Hence, double-application GB treatment of 10 mM + 10 mM reduced leaf NO_3^- content by more than 29% in comparison to untreated plants.

Lettuce growth

The double-application GB treatment (10 + 10 mM) resulted in significantly lower ($p = 0.014$) FW values in comparison to untreated plants only at a sampling time of 12 DAA (Figure 5A). Furthermore, the double-application of GB increased the DM accumulation in lettuce, while the leaf DM content was significantly ($p < 0.05$) higher in GB-treated plants at 5, 7, 12, and 14 DAA in comparison to untreated plants (Figure 5B).

Amino acid and mineral contents in lettuce

The root-applied GB affected the content and composition of amino acids in the lettuce DM (Table 1). There was significant interaction between time (DAA) and treatment (p < 0.05) in the contents of cysteine, glycine, aspartic acid, glutamic acid, leucine, and tryptophan, as well as the total content of amino acids, which increased following the GB treatment (Table 1). The difference between the GB-treated and untreated plants was highest at 10 DAA when the GB plants contained amino acids at 148 g kg^{-1} DM and untreated plants 133 g kg^{-1} DM.

The interaction between time (DAA) and treatment was significant (p < 0.05) in the total content of free amino acids, and especially in the contents of free histidine, asparagine, arginine, glutamine, glycine, and tyrosine (Table 2). The content of free amino acids in the GB-treated plants was lowest at 0 DAA (28 g kg^{-1} DM) and highest at 10 DAA (38 g kg^{-1} DM), whereas in untreated plants it was the lowest at 14 DAA (25 g kg^{-1} DM) and highest at 0 DAA (32 g kg^{-1} DM).

In general, the contents of minerals in the lettuce DM decreased after the GB application. Significant (p < 0.05) interaction between DAA and Trmt was observed in Ca, K, Mg, Mn, Na, P, and S contents, while the contents of Fe, Zn, N, and C were not significantly altered
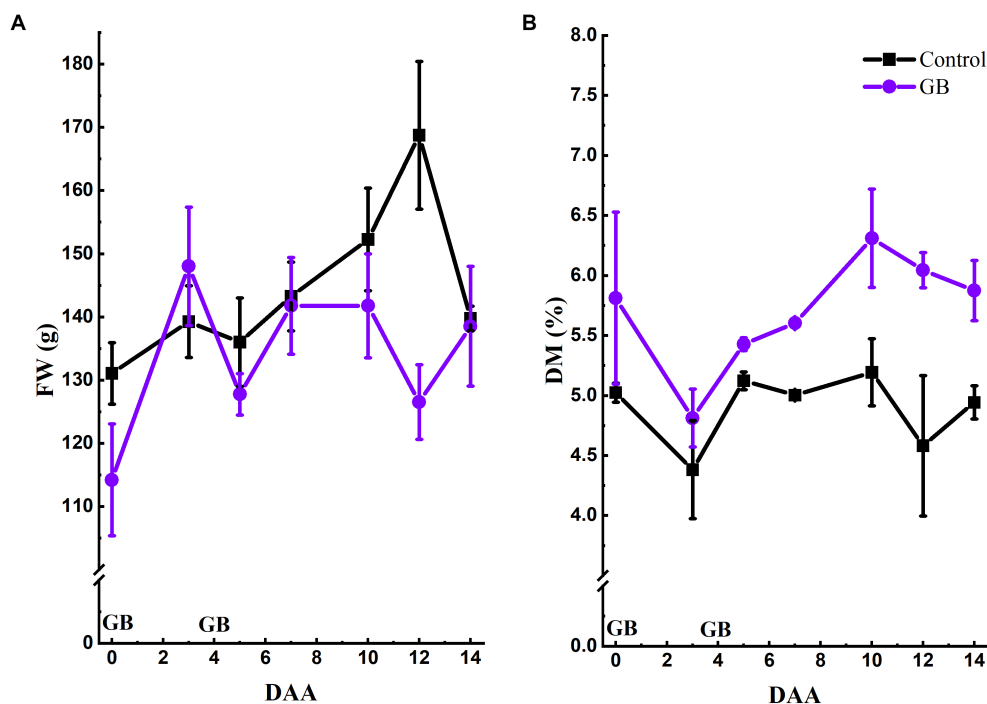
FIGURE 5
Effect of root-applied GB on lettuce fresh weight and dry matter content **(A)** Root-applied GB (10+10mM) reduced lettuce (cv. 'Frillice') fresh weight (FW) accumulation in comparison to untreated plants. **(B)** Root-applied GB increased the dry matter (DM) content of lettuce leaves. The application time of GB is marked above the X-axis. Data shown are means $\pm$ SE (**A**: N=10; **A**: N=4). GB=glycinebetaine; DAA=days after application.

(Table 3). The most marked change occurred in the content of K, which decreased over 43% following the double application (10 + 10 mM) GB treatment.

Discussion

Glycinebetaine and the edible quality of lettuce

The GB-treated lettuce accumulated root-applied GB in its leaves, whereas the untreated plants contained no GB, suggesting that lettuce does not synthesize endogenous GB as previously observed by Shams et al. (2016) and Jokinen et al. (2022). As the plants accumulated GB, the NO_3^- content in the leaves decreased. The marked decline in the leaf NO_3^- content began when the leaf GB content was $106 \mu g\,g^{-1}$ DM. Jokinen et al. (2022) observed that leaf GB content interacts negatively with leaf nitrate content, while a GB content of $90 \mu g\,g^{-1}$ DM was required to decrease the NO_3^- content. Decreased NO_3^- contents of lettuce contribute to consumers' health effects, and thus GB improves the edible quality of lettuce. Since GB itself is considered a compound with possible health benefits for humans (Schwab et al., 2002; Cho et al., 2006; Koistinen et al., 2019), its accumulation in lettuce leaves also contributes to the nutritional value of lettuce. Based on fresh lettuce, an edible dose of 100 g of fresh lettuce contained 0.7 mg of GB, the highest in this study.

The total content of amino acids in lettuce DM increased following the GB application, which is consistent with previous study by Jokinen et al. (2022). In terms of nutritive value, the nine

essential amino acids (histidine, threonine, lysine, methionine, valine, isoleucine, leucine, phenylalanine, and tryptophan), which cannot be synthesized in mammalian cells, are the most valuable nitrogenous compounds for human health (Davies and Jakeman, 2020). In our study the total content of the nine essential amino acids slightly increased in GB treated plants over the experiment period while in untreated plants it decreased. In plants, amino acids are produced through the NO_3^- assimilation process (Tischner, 2000). The increased amino acid content in GB-treated lettuce indicates that GB enhances NO_3^- assimilation and the activity of NO_3^- reductase (NR) and glutamine synthetase (GS) enzymes. Such effect of root-applied osmolytes (glucose and sucrose) were also previously reported in pak choi by Ma et al. (2018).

The contents of the minerals detected were lower in the GB treated plants at 10 DAA than in GB plants at 0 DAA. At a sampling time of 12 DAA, the GB-treated lettuce DM contained more Ca, Fe, Mg, Mn, Na, and C in comparison to untreated plants. However, the impact of GB on lettuce mineral uptake and use is rather unclear. Only the content of K was clearly reduced within GB-treated plants over time and remained a significantly lower level in the GB-treated plants in comparison to untreated plants throughout the experiment. Lettuce contained more DM after the GB application; hence, the reduction in the DM mineral contents did not directly contribute to reduced mineral content in edible fresh lettuce. Based on fresh lettuce, 100 g of fresh GB treated lettuce contained total N 9.7 mg at 0 DAA, and 11 mg at 10 DAA indicating that the reduction observed in NO_3^- content was not due to reduced N uptake. In addition, the content of C was slightly

TABLE 1 Effect of root-applied glycinebetaine (GB 10mM+10mM) on amino acids in lettuce leaves over time.

Amino acid content	Days after application								p-value		
	0		10		12		14		Time (T)	Trmt	T x Trmt
	Glycinebetaine treatment										
g kg⁻¹ DM	0mM	10+10mM	0mM	10+10mM	0mM	10+10mM	0mM	10+10mM	(df=3)	(df=1)	(df=3)
Cysteine	1.671[abc]	1.733[bc]	1.653[abc]	1.637[abc]	1.638[abc]	1.517[a]	1.808[c]	1.513[ab]	0.071	0.008	0.004
Histidine	2.562[a]	2.621[a]	2.475[a]	2.518[a]	2.627[a]	2.604[a]	2.670[a]	2.666[a]	0.004	0.535	0.740
Serine	6.553[a]	6.645[a]	6.452[a]	6.406[a]	6.650[a]	6.489[a]	6.677[a]	6.515[a]	0.180	0.265	0.418
Arginine	6.648[a]	6.736[a]	6.521[a]	6.499[a]	6.739[a]	7.024[a]	6.839[a]	6.926[a]	0.010	0.199	0.622
Glycine	6.924[ab]	7.123[ab]	6.904[ab]	6.749[a]	7.306[ab]	6.878[ab]	7.313[b]	7.013[ab]	0.025	0.032	0.040
Aspartic acid	13.138[ab]	13.165[ab]	12.304[a]	14.239[c]	12.784[ab]	13.498[bc]	12.826[ab]	13.443[bc]	0.911	0.001	0.002
Glutamic acid	37.626[a]	34.764[a]	33.923[a]	45.938[b]	33.148[a]	39.171[a]	32.940[a]	37.826[a]	0.010	0.001	0.001
Threonine	6.095[a]	6.215[a]	5.937[a]	6.017[a]	6.216[a]	6.094[a]	6.213[a]	6.160[a]	0.103	0.921	0.505
Alanine	7.417[a]	7.689[a]	7.346[a]	7.289[a]	7.715[a]	7.502[a]	7.623[a]	7.560[a]	0.014	0.815	0.086
Proline	5.849[ab]	6.090[ab]	5.823[a]	5.909[ab]	6.235[b]	6.03[ab]	6.204[ab]	6.089[ab]	0.007	0.977	0.060
Lysine	7.977[a]	8.426[a]	7.863[a]	8.019[a]	8.388[a]	8.268[a]	8.346[a]	8.324[a]	0.010	0.186	0.120
Tyrosine	3.019[ab]	3.473[bcd]	2.704[a]	3.588[bcd]	3.229[abcd]	3.843[d]	3.135[abc]	3.756[cd]	0.051	0.001	0.536
Methionine	2.565[a]	2.590[a]	2.570[a]	2.526[a]	2.704[a]	2.658[a]	2.798[a]	2.655[a]	0.017	0.218	0.562
Valine	7.195[a]	7.385[a]	7.061[a]	7.071[a]	7.420[a]	7.202[a]	7.429[a]	7.270[a]	0.031	0.525	0.172
Isoleucine	5.78[ab]	6.172[c]	5.702[a]	5.940[abc]	5.999[abc]	6.125[bc]	6.003[abc]	6.176[c]	0.014	0.001	0.409
Leucine	10.376[ab]	10.847[ab]	10.340[ab]	10.304[a]	11.017[b]	10.601[ab]	11.018[b]	10.694[ab]	0.007	0.486	0.033
Phenylalanine	6.542[ab]	6.736[ab]	6.491[ab]	6.328[a]	6.949[b]	6.528[ab]	6.997[b]	6.649[ab]	0.009	0.031	0.056
Tryptophan	1.405[a]	1.358[a]	1.449[a]	1.361[a]	1.517[a]	1.612[a]	1.350[a]	1.632[a]	0.040	0.209	0.041
Total	139.36[ab]	139.78[ab]	133.53[a]	148.35[b]	138.29[ab]	143.66[ab]	138.20[a]	142.88[ab]	0.907	0.001	0.021

Means sharing the same letter are not significantly different (Tukey HSD; $p < 0.05$); DM, dry matter; GB, glycinebetaine. Trmt, treatment; TxTrmt, time x treatment interaction; Total, the total concentration of amino acid; Data shown are means ± SE ($N = 4$).

increased in GB-treated plants based on FW. One edible dose of 100 g of fresh GB lettuce contained 2,039 mg C at 0 DAA and 2,220 mg at 12 DAA, whereas the yield of C in untreated plants was 1,742 and 1,527 mg 100 g⁻¹ FW, respectively. Furthermore, the DM content was a positively correlated with lettuce shelf life (Min et al., 2021), and thus our result indicates that GB may extend the shelf life of commercial lettuce.

Glycinebetaine in the commercial NFT-cultivation system

The GB disappeared from the nutrient solution within 3 to 5 days. Therefore, to continuously maintain low NO_3^- content in lettuce also requires repeated GB applications. Based on the results obtained in this study, GB application at 4-day intervals is recommendable. However, the GB uptake rate of lettuce may fluctuate over time and be influenced by environmental factors, plant maturity, and plant FW. In this study, plant responses to single GB applications at 4 mM and 7.5 mM concentration remained weak, whereas the double-application (10 mM+10 mM) resulted in similar leaf NO_3^- reduction previously reported by Jokinen et al. (2022). This result confirms the conclusion by Jokinen et al. (2022) that GB

reduces lettuce NO_3^- content in a dose-dependent manner. The number of plants in the treatment area, plant biomass, and plant age are likely significant factors regarding the strength of plant responses to applied GB. Thus, we conclude that the optimal GB concentration is farm-specific and dependent on the targeted reduction in NO_3^- content as well as the technical design of the application system.

Presumably, not all the applied GB was taken up by the plants. For example, it is known that some bacteria take up applied GB (Farwick et al., 1995). The GB applied may be reserved and released by the bacteria over time, which may partly explain why GB was found from leaves at 14 DAA even though it was last applied in the solution 10 days earlier. Regarding the practical use of GB, the sensory method for quick measurement of solution GB concentration would be useful.

In this experiment, the FW of the GB treated plants varied over time being significantly lower compared to control plants only on 12 DAA. Moreover, on 2 DAA the GB treated plants were heavier than control plants. Thus, the impact of GB on lettuce FW can be considered relatively slight in this experiment. In addition, the treatment did not lengthen the production period since all the plants reached the commercial size (100 g). This relatively slight growth retardation was observed probably because the plants were exposed

TABLE 2 Effect of root applied glycinebetaine (GB 10mM+10mM) on free amino acids in lettuce leaves over time.

Amino acid content	Days after application								P-value		
	0		10		12		14		Time (T)	Trmt	T x Trmt
	Glycinebetaine treatment										
g kg⁻¹ DM	0mM	10+10mM	0mM	10+10mM	0mM	10+10mM	0mM	10+10mM	(df=3)	(df=1)	(df=3)
Histidine*	0.157[a]	0.137[a]	0.140[a]	0.140[a]	0.121[a]	0.189[a]	0.121[a]	0.165[a]	0.791	0.050	0.040
Asparagine	1.915[a]	1.496[a]	1.446[b]	2.781[a]	1.199[a]	1.881[a]	1.316[a]	1.740[a]	0.005	0.001	0.001
Serine	0.796[a]	0.701[a]	0.787[a]	0.733[a]	0.686[a]	0.738[a]	0.656[a]	0.670[a]	0.153	0.513	0.373
Glutamine	21.758[ab]	18.599[a]	18.660[b]	27.726[a]	16.860[a]	22.430[ab]	16.205[a]	20.786[ab]	0.030	0.001	0.004
Arginine	0.892[abc]	0.749[ab]	0.787[ab]	0.917[abc]	0.720[a]	1.110[c]	0.715[a]	1.036[bc]	0.545	0.001	0.002
Glycine	0.077[a]	0.071[a]	0.074[a]	0.101[b]	0.074[a]	0.094[b]	0.073[a]	0.073[a]	0.001	0.001	0.001
Aspartic acid	0.558[c]	0.452[abc]	0.479[bc]	0.339[a]	0.428[abc]	0.362[ab]	0.406[ab]	0.377[ab]	0.002	0.001	0.292
Glutamic acid	2.006[c]	1.885[c]	1.911[c]	1.558[a]	1.454[bc]	1.851[a]	1.580[ab]	1.435[a]	0.001	0.001	0.053
Threonine*	0.611[a]	0.532[a]	0.574[a]	0.536[a]	0.509[a]	0.497[a]	0.479[a]	0.494[a]	0.064	0.256	0.579
Alanine	0.643[a]	0.576[a]	0.635[a]	0.517[a]	0.568[a]	0.558[a]	0.506[a]	0.567[a]	0.386	0.268	0.196
GABA	0.966[abc]	0.893[ab]	1.050[abc]	1.050[abc]	1.129[abc]	0.833[a]	1.311[c]	1.250[bc]	0.001	0.060	0.262
Proline	0.145[a]	0.132[a]	0.168[a]	0.204[a]	0.186[a]	0.181[a]	0.156[a]	0.187[a]	0.024	0.291	0.331
Lysine*	0.110[ab]	0.099[a]	0.134[abc]	0.127[abc]	0.167[c]	0.157[bc]	0.145[abc]	0.162[bc]	0.001	0.753	0.600
Tyrosine	0.278[a]	0.309[a]	0.240[a]	0.425[b]	0.247[a]	0.304[a]	0.237[a]	0.277[a]	0.003	0.001	0.001
Methionine*	0.024[a]	0.023[a]	0.021[a]	0.022[a]	0.027[a]	0.030[a]	0.025[a]	0.029[a]	0.119	0.483	0.848
Valine*	0.398[a]	0.328[a]	0.341[a]	0.332[a]	0.293[a]	0.334[a]	0.301[a]	0.330[a]	0.279	0.921	0.211
Isoleucine*	0.222[a]	0.195[a]	0.206[a]	0.209[a]	0.176[a]	0.226[a]	0.181[a]	0.212[a]	0.918	0.319	0.249
Leucine*	0.172[a]	0.155[a]	0.203[a]	0.191[a]	0.200[a]	0.216[a]	0.181[a]	0.211[a]	0.126	0.753	0.566
Phenylalanine*	0.154[a]	0.141[a]	0.163[a]	0.156[a]	0.167[a]	0.154[a]	0.166[a]	0.166[a]	0.400	0.286	0.919
Tryptophan*	0.052[a]	0.039[a]	0.037[a]	0.034[a]	0.033[a]	0.044[a]	0.032[a]	0.041[a]	0.212	0.774	0.091
Total	31.94[ab]	27.51[a]	28.06[a]	38.10[b]	25.64[a]	31.79[ab]	24.79[a]	30.21[ab]	0.048	0.005	0.008

Means sharing the same letter are not significantly different (Tukey HSD; $p < 0.05$); DM, dry matter; GABA, Gamma aminobutyric acid; GB, glycinebetaine. Trmt, treatment; T x Trmt, time x treatment interaction; *, essential amino acid; Total, total concentration of free amino acids; Data shown are means ± SE ($N = 4$).

to GB only during the last 6 growing days before harvest. The NO_3^- content was significantly reduced only after a 6-day treatment period with double-application of GB, indicating that a treatment period of 6 days is required in commercial production. The lower concentrations of GB tested in the two first experiments did not reduce leaf NO_3^- content. Assumably the GB concentration required to reach the targeted NO_3^- reduction vary depending on the total plant biomass on the treatment area.

The NO_3^- content in lettuce can also be regulated by reducing the concentration of NO_3–N in the nutrient solution before harvest (Santamaria et al., 1998). However, this method may reduce lettuce growth, increase tip burn in leaves, and shorten the shelf-life (Näkkilä et al., 2015). Even though GB can reduce phytomass accumulation, it can delay leaf senescence (Jokinen et al., 2022). Regulation of growth temperature (Santamaria et al., 2001) and optimizing light intensity (Liu et al., 2016; Zhang et al., 2018; Yi et al., 2020) are methods that use practical measures to regulate lettuce NO_3^- content and quality, but the useability of these methods is uncertain in the greenhouse. Accordingly, GB applied in the nutrient solution can be an alternative or supplemental method for lettuce quality control for greenhouse-grown lettuce.

Conclusion

This study demonstrated that GB provides a novel method for improving lettuce nutritive quality in commercial hydroponic NFT lettuce production by reducing leaf NO_3^- content and increasing the content of amino acids. The GB applied in the nutrient solution also increased the DM in lettuce. Lettuce containing more DM is more nutritious for humans. In addition, the increased DM content likely improves the shelf-life of lettuce. Some crop losses involved in high NO_3^- content can be avoided by GB application, which certainly benefits farmers and improves the efficiency throughout the production chain. These avoidable crop losses originated from practical methods for controlling the NO_3^- content by cutting off the oldest lettuce leaves during harvest, and rejection of the plants by traders due to exceeded NO_3^- content. However, the mode of action of root-applied GB on lettuce metabolism and leaf senescence is not yet known. This knowledge is essential, regarding the wider use of the method in commercial production and will therefore be a topic for future research.

TABLE 3 Effect of root applied glycinebetaine (GB 10mM+10mM) on mineral contents in lettuce leaves.

	Days after application										
	0		10		12		14		p-value		
Mineral content	GB treatment								Time (T)	Trmt	T x Trmt
gkg⁻¹ DM	0mM	10+10mM	0mM	10+10mM	0mM	10+10mM	0mM	10+10mM	(df=3)	(df=1)	(df=3)
Ca	9.490[cd]	9.991[d]	9.230[cd]	8.504[abc]	7.656[ab]	8.664[bc]	7.421[a]	8.608[bc]	<0.001	0.009	0.002
Fe	0.031[a]	0.035[ab]	0.034[ab]	0.032[a]	0.041[ab]	0.052[bc]	0.046[abc]	0.061[c]	<0.001	0.026	0.222
K	66.052[b]	69.277[bc]	75.828[c]	39.209[a]	60.731[b]	36.784[a]	62.505[b]	44.848[a]	<0.001	<0.001	<0.001
Mg	2.067[a]	3.484[d]	2.603[b]	2.776[bc]	1.810[a]	3.096[cd]	1.899[a]	2.855[bc]	<0.001	<0.001	<0.001
Mn	0.041[d]	0.053[e]	0.039[d]	0.034[bcd]	0.027[ab]	0.036[cd]	0.026[a]	0.029[abc]	<0.001	<0.001	<0.001
Na	0.811[d]	1.014[e]	0.711[cd]	0.501[a]	0.481[a]	0.677[bcd]	0.516[ab]	0.595[abc]	<0.001	0.012	<0.001
P	4.772[cd]	4.800[d]	4.781[cd]	3.574[ab]	3.998[b]	3.256[a]	4.167[bc]	3.173[a]	<0.001	<0.001	<0.001
S	2.231[b]	2.277[b]	2.279[b]	1.978[a]	2.251[b]	1.945[a]	2.231[b]	1.942[a]	0.013	<0.001	0.004
Zn	0.026[d]	0.024[cd]	0.021[bc]	0.020[ab]	0.021[bc]	0.021[bc]	0.016[a]	0.018[ab]	<0.001	0.528	0.293
N	39.150[bc]	38.000[abc]	38.850[bc]	34.125[ab]	38.925[bc]	33.025[a]	39.825[c]	33.150[a]	<0.001	0.117	0.089
C	346.75[ab]	351.00[ab]	330.50[a]	343.75[ab]	333.25[a]	367.25[b]	335.50[a]	351.00[ab]	0.001	0.134	0.111

Means sharing the same letter are not significantly different (Tukey HSD $p < 0.05$); DM, dry matter; Trmt, treatment; TxTrmt, time x treatment interaction; Ca, Calcium; Fe, Iron; K, Potassium; Mg, Magnesium; Mn, Manganese; Na, Sodium; P, Phosphorus; S, sulfhur; Zn, Zinc; N, Nitrogen; C, Carbon; Data shown are means ± SE ($N = 4$).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JU, KJ, and PM designed the experiments in collaboration with the commercial greenhouse. ME, KJ, IS, PM, and JU designed the chemical analyses conducted. JU conducted the experiments, performed the data work, and wrote the first draft of the manuscript. ME, PM, IS, and KJ provided revisions for the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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