SHORT-TERM COLD STORAGE REGULATES NITROGEN METABOLISM AND SAFETY IN ICEBERG LETTUCE (cv. TURNOVER)

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ABSTRACT

Understanding postharvest metabolic regulation during short-term cold storage is essential for maintaining the quality and chemical safety of leafy vegetables in regional supply chains. This study investigated physiological and metabolic responses of iceberg lettuce (*Lactuca sativa* L. cv. Turnover) during a three-day postharvest period at Laboratory of Tropical Horticultural Sciences, Tokyo University of Agriculture in July 2025. Freshly harvested iceberg lettuce was stored at 5°C for 72 hours. Changes in nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), nitrate reductase (NR), and nitrite reductase (NiR) activities, vitamin C, γ-aminobutyric acid (GABA), putrescine, and organic acids were monitored, and integrated using principal component analysis (PCA). Despite declining NR and NiR activities, NO_3^- and NO_2^- contents decreased steadily, indicating sustained internal nitrogen assimilation and improved food safety. PCA revealed a distinct metabolic transition on day 2, characterized by transient NH_4^+ accumulation, association with GABA and putrescine, and selective modulation of malic and citric acids, while succinic and fumaric acids remained relatively stable, indicating preservation of the core tricarboxylic acid (TCA) cycle. Vitamin C declined during this stress-responsive phase and partially stabilized as NH_4^+ levels decreased. Overall, short-term cold storage at 5 °C induces coordinated metabolic reprogramming rather than deterioration, promoting controlled nitrogen turnover and chemical safety of iceberg lettuce during early storage.

Key words: ammonium, GABA, lettuce, nitrate, postharvest quality.

INTRODUCTION

Iceberg lettuce (*Lactuca sativa* L. var. *capitata*) is a significant leafy vegetable in Japan, with Nagano Prefecture being the foremost production area, accounting for about 33% of the overall domestic output (MAFF 2024). Within this area, the Kawakami highland, situated at heights between roughly 1,100 and 1,500 meters above sea level, is officially identified as the top center for lettuce farming during summer and autumn due to its cool temperatures and suitable alpine conditions for cultivation (JMA 2025; MAFF 2024; Nagano Prefectural Government 2024). These elevated regions are commonly recognized for producing lettuce known for its exceptional texture and high nutritional value; however, these benefits may not always be preserved during post-harvest processes and distribution.

In Japan, iceberg lettuce sourced from elevated areas is generally delivered through a "highland-to-metropolitan" distribution system, which entails refrigerated transportation over considerable distances to major urban markets. Throughout this procedure, lettuce experiences mechanical harvesting impacts, quick cooling, and prolonged exposure to cool temperatures, all of which can disrupt cellular balance and trigger oxidative stress. Thus, ensuring biochemical stability during the initial stages of post-harvest logistics presents a notable challenge (Gross et al. 2016). This logistic model closely resembles those in Southeast Asia, where vegetables cultivated in highland zones – such as Dalat in Vietnam and the Cameron Highlands in Malaysia – are transported under refrigeration to lowland city markets, highlighting the importance of enhancing cold-chain management practices regionally (Altaki and Launio 2025).

In the context of quality and safety issues associated with leafy vegetables, effectively managing inorganic nitrogen components is vital. While the accumulation of nitrate (NO_3^-) is closely monitored due to its possible health hazards, it should be noted that NO_3^- does not remain chemically inactive after being harvested. Instead, it can be enzymatically converted into nitrite (NO_2^-) and then further into ammonium (NH_4^+) (Meyer and Stitt 2001). This conversion process is significantly affected by factors such as storage temperature and the conditions under which the vegetables are handled after harvest (Chandra et al. 2006; Chandra et al. 2008; Cintya et al. 2018; Xu et al. 2022). Importantly, the production of NO_2^- during storage raises more significant health concerns compared to NO_3^- itself. This emphasizes the importance of comprehending the transformations of nitrogen that occur after harvesting, rather than merely concentrating on the NO_3^- levels present at the time of harvest.

However, most current research measures NO_3^- levels as a static parameter at harvest, often neglecting the ongoing transformation of inorganic nitrogen ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$) during storage. This transformation is mediated by the sequential activities of nitrate reductase (NR) and nitrite reductase (NiR), yet the behavior of these enzymes during the early post-harvest period has not been adequately examined (Chandra et al. 2006; Chandra et al. 2008; Coronel et al. 2009; Xu et al. 2022; Gulyás et al. 2025). Importantly, reduced or imbalanced NR and NiR activities under low-temperature storage may result in the transient accumulation of NH_4^+, which represents a critical metabolic turning point rather than a passive end product. The first 72 hours after harvest – which includes harvesting, refrigerated transport, and initial distribution – constitute an essential, but underexplored window in which nitrogen assimilation, metabolic adjustment, and stress-responsive pathways may be rapidly reprogrammed.

Following inorganic nitrogen conversion, the accumulation of NH_4^+ during postharvest storage acts as a key metabolic signal that triggers downstream adjustments to maintain cellular homeostasis under low-temperature stress. Among these responses, γ-aminobutyric acid (GABA) and polyamines such as putrescine are closely associated with nitrogen rebalancing. GABA is a well-recognized stress-responsive metabolite that accumulates under mechanical injury and chilling and contributes to NH_4^+ assimilation, cytosolic pH regulation, and redox balance (Meyer and Stitt 2001). Likewise, putrescine, the simplest polyamine, is strongly linked to nitrogen availability and frequently increases under ammonium-rich conditions, supporting membrane stability and stress tolerance during postharvest storage (Gross et al. 2016).

In parallel, organic acids of the tricarboxylic acid (TCA) cycle serve as central integration points between carbon and nitrogen metabolism. Stress-induced modulation of malic and citric acids reflects metabolic flexibility and nitrogen redistribution, whereas the relative stability of succinic and fumaric acids indicates preservation of core respiratory function (Meyer and Stitt 2001). These metabolic adjustments are closely connected to cellular redox balance and antioxidant demand.

Within this framework, vitamin C functions as a primary antioxidant indicator of oxidative status and postharvest quality in leafy vegetables exposed to low temperature and mechanical stress (Smirnoff

2018). Although vitamin C depletion during cold storage has been widely reported (Cintya et al. 2018), its coordinated response with NH_4^+ accumulation and nitrogen-related metabolites during the early postharvest logistics period remains poorly understood.

To address these gaps, this study employed an experimental design that closely reflects commercial postharvest logistics. Iceberg lettuce cultivated in Nagano Prefecture was harvested at commercial maturity, transported under refrigeration to the Tokyo University of Agriculture, and stored at 5 °C in perforated polyethylene bags. Samples were analyzed immediately upon arrival at the laboratory (day 1), representing the post-logistics baseline condition. The remaining samples were maintained at 5 °C and analyzed over the subsequent two days, thereby encompassing the critical 72-hour postharvest logistics period.

Within this framework, three main contributions are presented. First, the study simultaneously monitors the complete inorganic nitrogen transformation ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$) together with its enzymatic regulators, NR and NiR, enabling an integrated evaluation of nitrogen turnover during early cold storage. Second, the accumulation of NH_4^+ is examined in relation to key nitrogen-responsive metabolites, including γ-aminobutyric acid (GABA) and putrescine, as well as selected tricarboxylic acid (TCA) cycle – related organic acids, thereby capturing coordinated carbon–nitrogen metabolic adjustments under low-temperature stress. Third, by defining the initial 72 hours after harvest as a critical logistics window, this work enhances the practical relevance of laboratory observations for cold-chain management in Japan and comparable highland-to-lowland supply systems in Southeast Asia (Altaki and Launio 2025; Gross et al. 2016).

In summary, this research investigated whether short-term storage at 5 °C during the critical 72-hour logistics window can maintain metabolic stability, nutritional quality, and chemical safety in iceberg lettuce, through the integrated assessment of nitrogen conversion, stress-related metabolites (GABA and putrescine), TCA cycle–associated organic acids, and vitamin C dynamics. The findings provide practical insights for improving cold-chain management in Japan and regional vegetable supply chains across Southeast Asia.

MATERIALS AND METHODS

Iceberg lettuce (*Lactuca sativa* L.) cultivar ‘Turnover’ was harvested at commercial maturity in July 2025 from Kawakami, Nagano Prefecture, Japan. Immediately after harvest, the lettuce was transported under refrigerated conditions to the Laboratory of Tropical Horticultural Sciences, Tokyo University of Agriculture. Upon arrival, samples were taken as the post-logistics baseline (day 1), and the remaining lettuces were stored at 5 °C in perforated polyethylene bags

Subsequently, samples were collected at 24 h intervals over the following two days. At each sampling point, the third fully expanded leaves were selected as representative tissues and divided for different analytical purposes. One portion of the fresh leaf tissue was used on the same day for the determination of inorganic nitrogen components (nitrate, nitrite, and ammonium), vitamin C content, and the activities of nitrate reductase and nitrite reductase.

The remaining portion of the leaf tissue was prepared for metabolite analysis by gas chromatography–mass spectrometry (GC–MS). Fresh tissues were finely chopped and immediately frozen with liquid nitrogen to rapidly quench metabolic activity, then ground into a fine powder using a pre-chilled mortar and pestle under liquid nitrogen. The resulting powders were transferred into cryogenic tubes and stored at −80 °C until subsequent extraction and GC–MS analysis of γ-aminobutyric acid (GABA), putrescine, and tricarboxylic acid (TCA) cycle–related organic acids.

Nitrate and nitrite content. The determination of NO_3^- and NO_2^- levels in vegetables was carried out using a reflectometric method following the application of a reducing agent and the Griess reaction (Merino 2009). This was performed with a reflectometer (RQ-flex Plus 10, Merck Inc., Darmstadt, Germany) in accordance with the research conducted by Nguyen et al. (2025). The concentrations of NO_3^- and NO_2^- were recorded as milligrams of NO_3^- and NO_2^- per kilogram of fresh weight (FW), respectively.

Ammonium content. The NH_4^+ concentration in the crude extract was assessed by utilizing a modified Berthelot reaction, following the procedure established by Weatherburn in 1967. A sample weighing 0.5 grams of lettuce leaf was powdered using a mortar and pestle in the presence of 3 mL of 0.3 mM sulfuric acid, while ensuring the pH remained at 3.5. The homogenized mixture was then subjected to centrifugation at a speed of 12,000 rounds per minute for a period of 10 minutes at a temperature of 25 °C. After centrifugation, 200 µL of the transparent supernatant was mixed with 3.8 mL of 0.3 mM sulfuric acid, resulting in a total solution volume of 4 mL. For the subsequent colorimetric reaction, 0.5 mL of solution A, which consists of 5 grams of phenol and 25 milligrams of nitroprusside dissolved in 100 mL of water, was incorporated, followed by the addition of 0.5 mL of solution B. Solution B was produced by combining 40 mL of 5% sodium hypochlorite with 2.5 grams of NaOH and diluting this mixture to a final volume of 100 mL with distilled water. The combined solution was left to incubate with gentle agitation in a water bath maintained at 37 °C for a duration of 20 minutes. The absorbance was subsequently measured at 625 nm against a control sample that lacked the extract. NH_4^+ levels were estimated using an extinction coefficient of 3.646 $\mu mol^{-1} \cdot cm^{-1}$ and were expressed as mg NH_4^+ per kilogram of FW.

Vitamin C content. The content of vitamin C (ascorbic acid) in the vegetables was determined through a reflectometric technique, employing a reflectometer (RQ-flex Plus 10, Merck Inc., Darmstadt, Germany) in accordance with previous research of Nguyen et al. (2025). The ascorbic acid concentration was reported as milligrams per kilogram of FW.

Nitrate reductase activity. The assessment of nitrate reductase activity (NRA) was conducted according to the guidelines set forth by Neyra and Hageman in 1974, with adjustments as noted by Segura in 1990. A sample of 0.5 grams from the third leaves was acquired, finely minced, and subsequently introduced to 5 mL of incubation solution with a pH of 7.5. This procedure took place in a dark setting at ambient temperature, specifically at 28 ± 2 °C, over a period of 60 minutes. The incubation solution was composed of 1 mL of 0.1 M KNO_3, 3.75 mL of a blend containing 0.1 M K_2HPO_4 and KH_2PO_4, along with 0.25 mL of a 1% (v/v) n-propanol solution. Following the incubation phase, new solutions were prepared by mixing 2 mL of the incubation solution with 1 mL of nitrite reactive, which included Sulfanilamide at a concentration of 1% (w/v) in 3 M HCl and 0.02% N-(1-naftil) ethylene di-amine di-hydrochloride, alongside a control consisting of 2 mL of the incubation solution and 1 mL of distilled water. This combination was incubated in the dark for 15 minutes to facilitate color formation. The absorbance readings were obtained via a spectrophotometer set at a wavelength of 540 nm to establish a nitrites standard curve using a 10 ppm N solution (as $NaNO_2$). The findings were reported as one unit of NR activity in micromoles of NO_2^- per hour per gram of FW.

Nitrite reductase activity. The evaluation of nitrite reductase activity (NiRA) was performed in accordance with the protocol established by Wray and Fido in 1990, which utilized dithionite-reduced methyl viologen as an artificial electron donor.

Approximately 0.5 grams of the fresh specimen were pulverized in a pre-cooled mortar with a pestle, accompanied by 5 mL of distilled water. Following this, the resultant extract underwent centrifugation at a rate of 12,000 rounds per minute for a duration of 10 minutes at a temperature of 25°C, utilizing the TOMY MX-307 high-speed refrigerated microcentrifuge (Japan). For the assay procedures, 50 µL of the extract was mixed with 125 µL of a 50 mM potassium phosphate buffer adjusted to a pH of 7.5,

along with 125 µL of potassium nitrite (2.5 mM KNO_2) and 200 µL of a freshly prepared 20 mM sodium dithionite ($Na_2S_2O_4$) solution in 290 mM sodium bicarbonate. The reaction commenced upon the addition of sodium dithionite, succeeded by the incorporation of 125 µL of methyl viologen (3 mM methyl viologen), which resulted in the formation of a blue hue. Control samples, denoted as blanks, encompassed all assay components apart from methyl viologen. After permitting an incubation period of 15 minutes at 25°C in open tubes, the reaction process was terminated by thoroughly mixing the tube contents until both dithionite and the reduced methyl viologen were oxidized, as indicated by the loss of the blue coloration of the reduced dye. A 0.1 mL aliquot of the reaction mixture was then diluted with 2.9 mL of distilled water, in addition to 1 mL of each of the 1% (w/v) sulphanilamide solution in 3 M HCl and 0.02% (w/v) N-(1-naphthyl) ethylene-diamine dihydrochloride. This combination was incubated for a further 15 minutes. The absorbance was measured at a wavelength of 540 nm using an appropriate blank in a spectrophotometer, which enabled the determination of NiR activity. One unit of NiR activity was specified as the production of 1 µmol of NO_2^- per hour per gram of FW.

Stress-related metabolites and TCA cycle related organic acids. 100 mg of homogenized lettuce powder was used to prepare each sample. Each sample was combined with one zirconia bead and 250 µL of methanol, then mixed in a mixer mill MM400 for 2 minutes at 27 Hz. After that, 250 µL of chloroform was added, and then incubated in an Eppendorf thermomixer F2.0 for 3 minutes at 37 °C and 1200 rpm. Subsequently, 50 µL of standard solution (composed of 2 mg of ribitol in 1 mL of ultra-pure water) and 175 µL of ultra-pure water were added to each sample then vortexed until completely mixed by VORTEX GENIE 2. After this, the samples were centrifuged at 1,200 rpm for 10 minutes at 25 °C TOMY MX-307. 80 µL of supernatant was transferred into a new 1.5 mL tube and centrifugal evaporated for 2 hours in EYELA CVE-3110. Samples were then placed in a freeze-dryer machine EYELA FDM-100 at −45 °C overnight.

Following lyophilization, each sample received 40 µL of methoxamine hydrochloride (MACl) (composed of 20 mg of MACl mixed with 1 mL of pyridine solution), and then were placed back into the Eppendorf thermomixer F2.0 for 90 minutes at 37°C. Next, 50 µL of N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) was added and then centrifuged again in the Nichiryo C1008-B and subsequently placed back into the Eppendorf thermomixer F2. 0 for an additional 30 minutes at 37 °C. Next, 50 µL of the solution was extracted and transferred into small bottles designed for the Gas Chromatograph-Mass Spectrometer (GCMS) procedure in Shimadzu GC-2010 coupled with GCMS-QP 2010 Plus.

Statistical analysis. All data were analyzed using one-way analysis of variance (ANOVA) with storage time (day 1, day 2, and day 3) as the independent factor, followed by Tukey's multiple comparison test to determine significant differences among means at $p < 0.05$. Principal component analysis (PCA) was applied to integrated metabolic variables, including inorganic nitrogen components, enzyme activities, stress-related metabolites, organic acids, and vitamin C, to evaluate coordinated metabolic responses during short-term cold storage. All statistical analyses and graphical visualizations were performed using R software (version 4.4.1) and RStudio (version 2025.05.1+513).

RESULTS AND DISCUSSION

Visual change during storage. A noticeable change was observed in the appearance of iceberg lettuce from day one to day three (Fig. 1). The fresh samples retained a solid texture and substantial turgor on day one, while those collected on day two showed initial signs of wilting and a decrease in crunchiness. By day three, significant leaf curling, softening of the tissue, and a lackluster surface became apparent, signifying an aging symptom.

These changes are characteristic of the stress that occurs after harvest during cold storage, where the lack of light inhibits photosynthesis and accelerates senescence processes, including the deterioration of membranes and oxidative damage (Toivonen and Brummell 2008).

Figure 1. Visual changes during storage of iceberge lettuce (cv. Turnover)

In leafy greens such as lettuce, the absence of light, as well as cold temperature, quickly impacts the metabolism of carbon and nitrogen, initiating physiological reactions that occur prior to biochemical alterations in nitrogen compounds and antioxidant systems (Canetti et al. 2002).

Nitrogen metabolism and antioxidant responses during storage. This section collectively describes the coordinated changes in inorganic nitrogen compounds (NO_3^-, NO_2^-, NH_4^+), nitrogen metabolic enzymes (NR and NiR), and antioxidant levels (vitamin C) in iceberg lettuce while it is kept in cold storage (Fig. 2).

Changes in nitrate, nitrite and their relationship with nitrate reductase and nitrite reductase activities. NO_3^- content declined steadily from day one to day three (Fig. 2A), which was accompanied by a notable reduction in the activity of NR (Fig. 2D). A similar declining trend was observed in NO_2^- levels and NiRA (Fig. 2B and 2E).

The simultaneous reductions in both NO_3^- levels and NRA can be mechanistically linked to the lack of light and coldness during the storage period. NR is an enzyme regulated by light, and its activity relies on reducing power generated through photosynthetically. Under dark storage, NR becomes rapidly inactivated through phosphorylation, often followed by a decrease in protein stability and proteolytic degradation, resulting in suppressed NO_3^- reduction capacity (Kaiser and Huber 2001). From a food safety perspective, the progressive decline in NO_3^- content observed during storage is favorable, as high NO_3^- intake from leafy vegetables is considered a major dietary exposure route (Santamaria 2006).

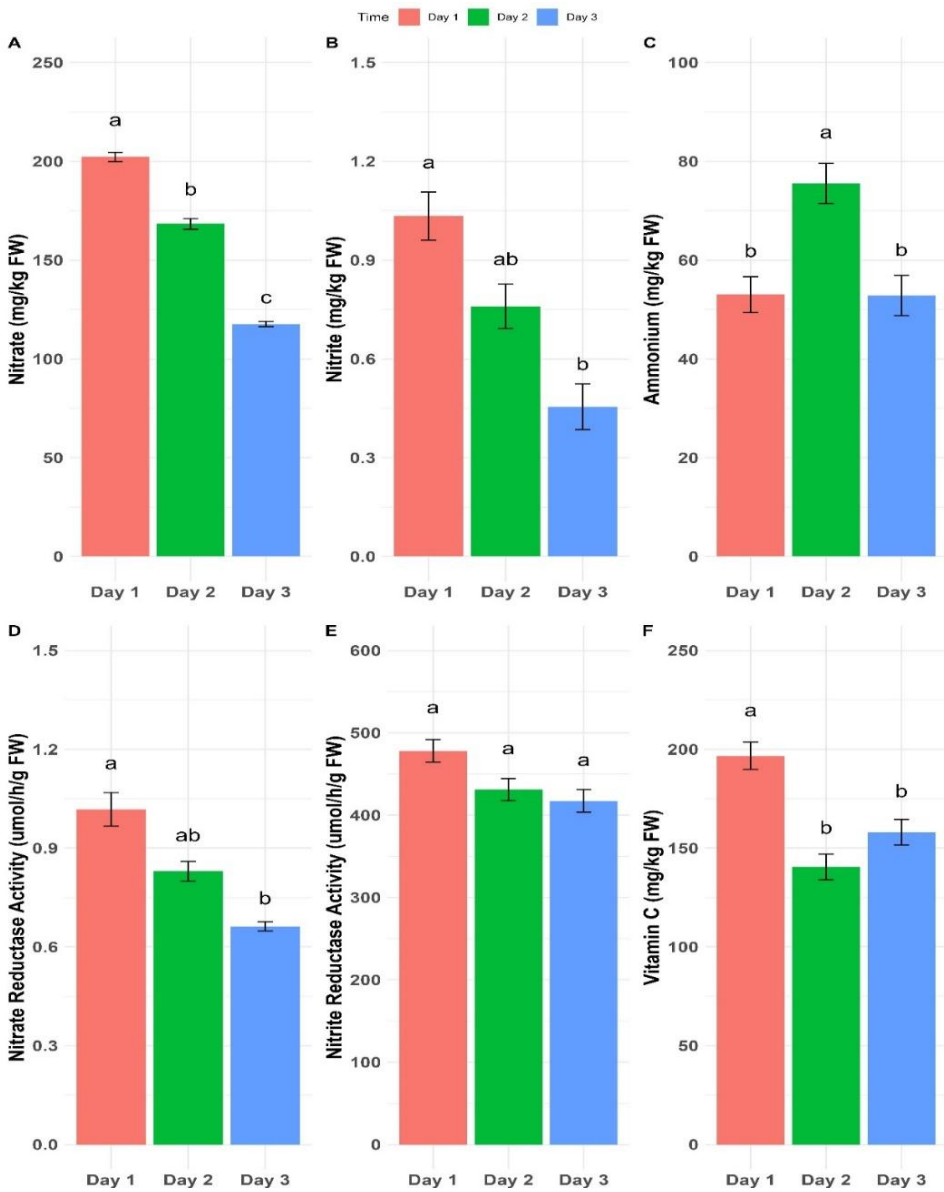

Figure 2. The effect of storage time on (A) Nitrate content, (B) Nitrate Reductase Activity, (C) Nitrite content, (D) Nitrite Reductase Activity, (E) Ammonium content and (F) Vitamin C content in iceberg lettuce (cv. Turnover)

Values are expressed as means (n = 3). Error bars represent standard error of the means. Different lowercase letters indicate significant differences according to the one-way ANOVA followed by the Tukey test ($p < 0.05$).

Similarly, NiRA decreased during the storage period, indicating a lower availability of NO_2^- substrate and a reduced metabolic requirement. In cold and dark environments, the coordinated downregulation of NR and NiR prevents the accumulation of NO_2^-. NO_2^- accumulation is of particular concern due to its higher toxicity compared with NO_3^-, emphasizing the importance of maintaining efficient NO_2^- reduction in leafy vegetables (Santamaria 2006). This observation clarifies why NO_2^-

levels did not rise in this study, in contrast to findings reported during storage at ambient temperature (Cintya et al. 2018).

The simultaneous reduction of NR, NiR, NO_3^-, and NO_2^- illustrates a strong metabolic linkage within the NO_3^- assimilation pathway. Similar patterns have been reported in postharvest leafy tissues stored under dark conditions, where reduced nitrate-reducing enzyme activities limit both NO_3^- utilization and NO_2^- formation (Xu et al. 2022).

Transient accumulation of ammonium and its physiological significance. A unique trend was identified concerning NH_4^+, which exhibited a significant increase on day two, followed by a decrease on day three (Fig. 2C). This temporary rise indicates a metabolic imbalance between nitrogen reduction and assimilation during the initial storage phase.

The notable spike observed on day two may be associated with postharvest physiological stress, which promotes proteolysis and subsequent amino acid deamination induced by harvest injury and cold shock (Amodio et al. 2018). Simultaneously, the residual activity of NR and NiR during the early storage period may allow continue nitrogen reduction, potentially contributing to produce NH_4^+ (Kaiser and Huber 2001; Chandra et al. 2006). Together, these processes can induce a transient metabolic imbalance in which NH_4^+ generation may exceed the capacity of NH_4^+ assimilation via the GS/GOGAT pathway, thereby promoting NH_4^+ accumulation and postharvest physiological stress (Amodio et al. 2018; Forde and Lea 2007; Hachiya and Noguchi 2011).

The observed reduction in NH_4^+ levels on the third day is likely due to a diminished upstream supply originating from NO_3^- and NO_2^-, as extended storage is known to further suppress the activities of NR and NiR (Kaiser and Huber, 2001), together with a general decline in nitrogen metabolic capacity reported in stored lettuce (Chandra et al. 2006). This decrease may also indicate a combination of ammonia loss through volatilization (Amodio et al. 2018) and the gradual exhaustion of easily accessible protein reserves as senescence progresses (Masclaux-Daubresse et al. 2010). At the same time, the reassimilation of NH_4^+ through the GS/GOGAT pathway is essential for preventing NH_4^+ toxicity and sustaining cellular stability, particularly under conditions where nitrogen metabolism becomes increasingly constrained during postharvest senescence (Bittsánszky et al. 2015; Hachiya and Noguchi 2011). In summary, this trend suggests a shift from active nitrogen metabolism to a gradual metabolic shutdown, which is a characteristic of the late postharvest phase in leafy vegetables (Chandra et al. 2008; Gross et al. 2016).

Interaction between ammonium accumulation and vitamin C dynamics. Vitamin C content declined sharply from day one to day two, coinciding with the peak in NH_4^+ concentration, and then showed a slight recovery trend on day three (Fig. 2F), although the increase was not statistically significant.

This negative correlation implies that vitamin C may contribute to protective responses under elevated NH_4^+ conditions. It is established that high amounts of NH_4^+ can lead to serious physiological stress that disrupts pH gradients across membranes and increases the production of reactive oxygen species (ROS) (Bittsánszky et al. 2015; Li et al. 2021). To counteract ammonium-induced oxidative stress, plants primarily depend on ascorbic acid as a key ROS scavenger, leading to its swift depletion (Foyer and Noctor 2011; Smirnoff 2018). Therefore, the significant drop in vitamin C observed on day two likely indicates a rapid consumption of ascorbic acid due to oxidative stress caused by NH_4^+.

As the levels of NH_4^+ decreased on day three, oxidative stress might have been lessened to some extent, potentially allowing vitamin C levels to stabilize or slightly recover due to a reduced antioxidant demand, despite limited de novo biosynthesis under dark storage conditions. This partial recuperation may reflect a physiological adjustment during later postharvest stages aimed at re-establishing cellular redox equilibrium as the impacts of proteolysis and NH_4^+ toxicity diminish (Foyer and Noctor 2011;

Smirnoff 2018). Similar interactions between ammonium-induced oxidative stress and antioxidant depletion have been documented under nitrogen imbalance conditions in plants (Li et al. 2021).

Changes in amino acid, polyamine and organic acids during cold storage. Significant changes over time were noted in metabolites related to stress and organic acids in iceberg lettuce during cold storage (Fig. 3).

The content of γ-aminobutyric acid (GABA) showed a substantial increase from day one to day two ($p < 0.05$) and maintained a significantly elevated level at day three when compared to day one (Fig. 3A). Putrescine demonstrated a notable temporary accumulation, reaching its peak on day two before experiencing a sharp decline by day three (Fig. 3B).

Among the intermediates of the tricarboxylic acid (TCA) cycle, succinic acid and fumaric acid showed no significant differences among the three storage days (Fig. 3C and 3D). In contrast, malic acid content decreased progressively from day one to day three, with significantly lower values on day three compared to day one (Fig. 3E).

Citric acid exhibited a distinct transient pattern, with a significant increase on day two followed by a decrease on day three, although day three values remained higher than those measured on day one (Fig. 3F).

The metabolic patterns observed indicate that iceberg lettuce underwent a transient but coordinated metabolic adjustment during cold storage, with day two representing a critical phase of physiological response rather than progressive deterioration (Fig. 3).

The sharp increase in GABA and putrescine on day two reflects a rapid activation of stress-responsive nitrogen metabolism pathways. GABA accumulation is widely recognized as an early response to abiotic stress, functioning both as a metabolite linking carbon and nitrogen metabolism and as a signaling molecule involved in stress responses (Forde and Lea 2007; Ramesh et al. 2015). Similarly, putrescine accumulation is associated with enhanced polyamine biosynthesis under stress conditions, contributing to membrane stabilization and cellular protection (Groppa and Benavides 2008).

The subsequent decline of both metabolites on day three suggests that this response was transient and regulated, consistent with an adaptive adjustment to cold storage rather than irreversible metabolic disruption.

A notable result is the absence of significant differences in succinic and fumaric acid contents across the three storage days. As both metabolites are core intermediates of the TCA cycle, their stability indicates that mitochondrial respiratory metabolism remained largely intact during short-term cold storage, suggesting maintenance of basal respiratory activity under appropriate postharvest handling conditions (Cantwell and Suslow 2002; Gross et al. 2016). In contrast, the dynamic changes observed for malic and citric acids indicate flexible regulation of specific TCA cycle branches, allowing metabolic adjustment without compromising basal respiration.

The metabolic configuration observed on day two provides a mechanistic basis for the transient increase in NH_4^+ content observed in Figure 2. NH_4^+ accumulation is a sensitive indicator of postharvest physiological stress rather than a symptom of metabolic failure (Amodio et al. 2018). In the present study, the NH_4^+ pulse coincided with maximal levels of GABA and putrescine, suggesting that excess NH_4^+ was, at least in part, actively reassimilated and diverted into nitrogen-rich protective metabolites. Such metabolic redirection is consistent with the established roles of GABA and polyamines in linking nitrogen and carbon metabolism and in enhancing cellular protection under stress conditions (Forde and Lea 2007; Groppa and Benavides 2008; Ramesh et al. 2015).

The stability of succinic and fumaric acids further suggests that NH_4^+ detoxification did not disrupt basal respiratory metabolism, supporting the concept of a controlled metabolic stress response (Bittsánszky et al. 2015; Gross et al. 2016).

The metabolic adjustments are closely linked to the changes in NO_3^-, NO_2^-, (Fig. 3) and the activities of NR and NiR (Fig. 2). The coordinated accumulation of organic acids and nitrogenous metabolites on day two suggests that residual NRA and NiRA remained functional, allowing continued NO_3^- and NO_2^- reduction despite cold-induced stress.

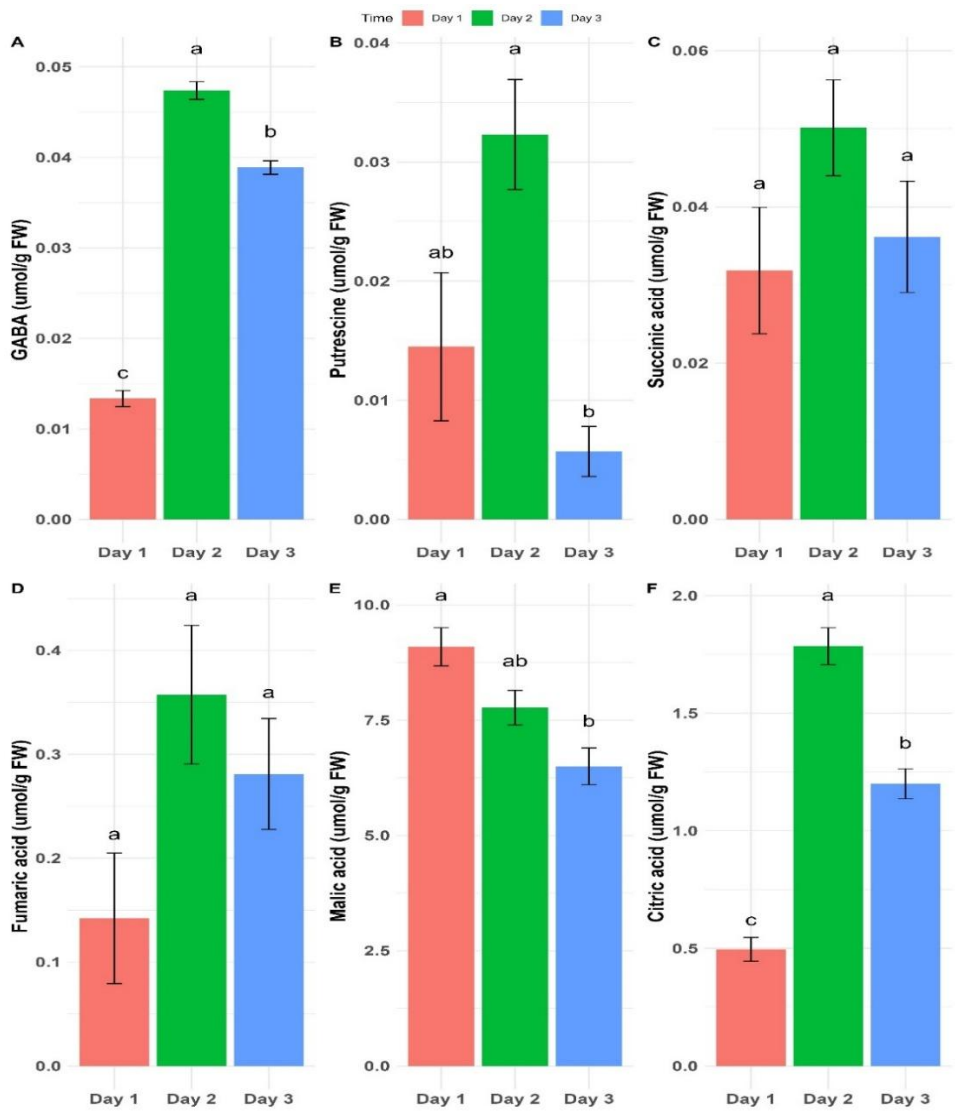

Figure 3. The effect of storage time on (A) GABA, (B) Putrescine, (C) Succinic acid, (D) Fumaric acid, (E) Malic acid and (F) Citric acid in iceberg lettuce (cv. Turnover)

Values are expressed as means (n = 3). Error bars represent standard error of the means. Different lowercase letters indicate significant differences according to the one-way ANOVA followed by the Tukey test ($p < 0.05$).

Previous studies have demonstrated that NRA can be modulated through post-translational regulation under stress conditions, depending on the availability of carbon skeletons and reducing power (Kaiser and Huber 2001; Meyer and Stitt 2001). The observed metabolic balance in the present study likely prevented excessive NO_2^- accumulation, a phenomenon frequently associated with postharvest metabolic impairment (Santamaria 2006; Xu et al. 2022).

The progressive decrease in vitamin C content observed in Figure 2 can be interpreted in the context of the metabolic responses (Fig. 3). Ascorbate plays a central role in cellular redox homeostasis and is actively consumed during stress adaptation (Foyer and Noctor 2011; Smirnoff 2018). The peak metabolic activity on day two, characterized by enhanced nitrogen assimilation and organic acid turnover, likely increased cellular oxidative pressure and antioxidant demand, thereby contributing to vitamin C depletion.

Similar trends have been reported in leafy vegetables during cold storage, where vitamin C loss accompanies metabolic adjustment under refrigerated conditions rather than uncontrolled senescence (Cintya et al. 2018; Gross et al. 2016).

The short-lived metabolic pulse observed in this study reflects a rapid but controlled physiological response following harvest. The maintenance of respiratory stability and nitrogen assimilation capacity supports the effectiveness of the postharvest handling strategy applied to iceberg lettuce (Cantwell and Suslow 2002; Gross et al. 2016).

The noted alterations in GABA, putrescine, and organic acids provide significant mechanistic insight into the nitrogen absorption processes of iceberg lettuce during cold storage. Previous findings indicated a coordinated reduction in the levels of NO_3^- and NO_2^-, which was coupled with maintained activities of NR and NiR, alongside a temporary increase in NH_4^+ observed by day two. NH_4^+ serves as a vital metabolic convergence point, as it requires prompt reassimilation to prevent cellular toxicity (Amodio et al. 2018; Bittsánszky et al. 2015).

The prominent accumulation of GABA from day two onwards strongly suggests the engagement of the GABA shunt, a key metabolic route linking NH_4^+ assimilation with carbon metabolism. GABA is produced from glutamate, a central product of NH_4^+ assimilation via the GS/GOGAT cycle, and its accumulation has been widely reported as a stress-responsive mechanism contributing to pH regulation, redox balance, and the integration of carbon and nitrogen metabolism (Forde and Lea 2007; Li et al. 2021). The sustained presence of GABA on day three indicates that iceberg lettuce did not undergo irreversible metabolic disruption but instead entered a phase of metabolic stabilization during cold storage.

Putrescine exhibited a notable transitory increase on day two, in alignment with heightened NH_4^+ levels and the initial restructuring of carbon metabolism. Polyamines like putrescine are recognized for their rapid accumulation under abiotic stress, contributing to protective functions such as membrane stabilization and scavenging of reactive oxygen species (Han et al. 2025). The following decrease in putrescine by day three implies that the initial stress response was effectively resolved, in agreement with the lack of significant senescence signs.

Putrescine exhibited a notable transitory increase on day two, in alignment with heightened NH_4^+ levels and the initial restructuring of carbon metabolism. Polyamines such as putrescine are known to rapidly accumulate under abiotic stress and contribute to cellular protection, including membrane stabilization and mitigation of oxidative damage (Groppa and Benavides 2008; Han et al. 2025). The subsequent decline in putrescine levels by day three suggests that the initial stress response was effectively resolved, consistent with the absence of pronounced senescence symptoms.

The redistribution of TCA cycle intermediates further reinforces this viewpoint. The rises in citric, succinic, and fumaric acids by day two may reflect an increased requirement for carbon skeletons to facilitate nitrogen reassimilation and other metabolic processes associated with stress. The

accumulation of citrate has been linked to a heightened flow through the TCA cycle during NH_4^+ assimilation, which provides energy and carbon scaffolding for amino acid production (Masclaux-Daubresse et al. 2010; Li et al. 2021). In contrast, the gradual reduction of malic acid implies a transition from a high-respiration, fresh-harvest metabolic condition toward a more organized and energy-efficient metabolism during storage (Cantwell and Suslow 2002; Gross et al. 2016).

From a postharvest viewpoint, these results illustrate that iceberg lettuce has the ability to integrate nitrogen metabolism with carbon metabolism that responds to stress, enabling it to sustain physiological stability during cold storage. This metabolic synchronization is likely significant in postponing degradation processes related to senescence, which are frequently linked to unregulated nitrogen remobilization and the breakdown of nucleic acids (Canetti et al. 2002; Masclaux-Daubresse et al. 2010). This finding underlines the efficacy of cold-chain management in maintaining both nitrogen safety and metabolic integrity in Iceberg lettuce, in alignment with recognized principles of postharvest handling (Cantwell and Suslow 2002; Gross et al. 2016).

Principal component analysis of nitrogen metabolism. The first principal component analysis (PCA 1) concerning nitrogen metabolism, which includes nitrate (NO_3^-), nitrite (NO_2^-), nitrate reductase activity (NRA), nitrite reductase activity (NiRA), and ammonium (NH_4^+), distinctly classified lettuce samples based on the length of storage (Fig. 4). The initial two principal components (Dim1 and Dim2) accounted for 64.5% and 21.0% of the overall variance, respectively, indicating a strong differentiation in storage time.

Dim1 primarily represented the general ability for NO_3^- reduction. Samples from Day 1 were located on the positive axis of Dim1 and were closely linked to higher levels of NO_3^- and NO_2^- as well as increased NRA and NiRA activities, indicating an efficient NO_3^- assimilation system. Conversely, Day 3 samples were found on the negative side of Dim1, which correlates with a substantial decrease in NO_3^- reduction capability due to extended storage time.

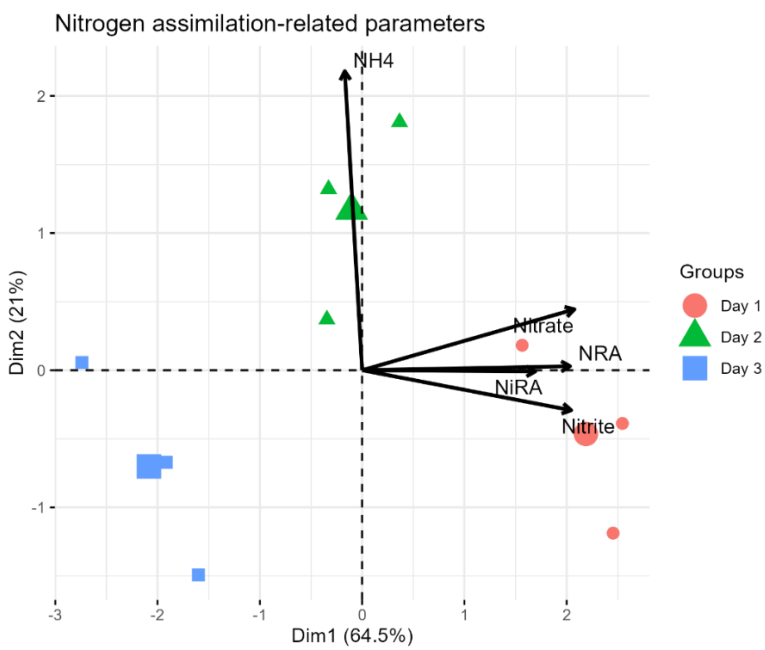

Figure 4. Principal component analysis of nitrogen metabolites during storage of iceberg lettuce (cv. Turnover)

Samples from Day 2 created a unique cluster that was mainly differentiated along Dim2, influenced by heightened NH_4^+ accumulation and changes in the relationship between NRA and NiRA. This arrangement signifies that the metabolic alterations observed on Day 2 are not a straightforward progression from Day 1 to Day 3, but rather indicate a temporary metabolic phase.

In summary, the PCA indicates a non-linear pathway of nitrogen metabolism throughout the storage period, with Day 2 representing a brief metabolic surge. The distinct but intermediate position of the Day 2 samples points to an immediate physiological reaction shortly after the start of storage, confirming metabolic reorganization induced by postharvest stress (Cantwell and Suslow 2002; Gross et al. 2016). The strong correlation between NH_4^+ accumulation and Day 2 reinforces the concept of NH_4^+ as a sensitive marker for postharvest stress, as suggested by Amodio and coworkers (2018).

Integration of the PCA with the temporal patterns observed in Figure 2 suggests that NH_4^+ accumulation at Day 2 arises from multiple internal sources, including not only ongoing NO_3^- and NO_2^- reduction but also enhanced protein and amino acid catabolism triggered by postharvest stress. Dark- and stress-induced proteolysis has been reported to release free NH_4^+ during early senescence and metabolic adjustment (Masclaux-Daubresse et al. 2010). Although NRA and NiRA remain active, limitations in carbon skeleton availability and energy supply likely constrain further incorporation of NH_4^+ into amino acids, leading to its transient accumulation (Hirel et al. 2007).

Principal component analysis of metabolites stress and antioxidant status. The second principal component analysis (PCA 2) included vitamin C, organic acids (citric, malic, succinic, and fumaric acids), GABA, and putrescine (Fig. 5). The first two components explained 62.4% (Dim1) and 18.0% (Dim2) of the total variance, accounting for 80.4% cumulatively, and clearly separated samples according to storage duration.

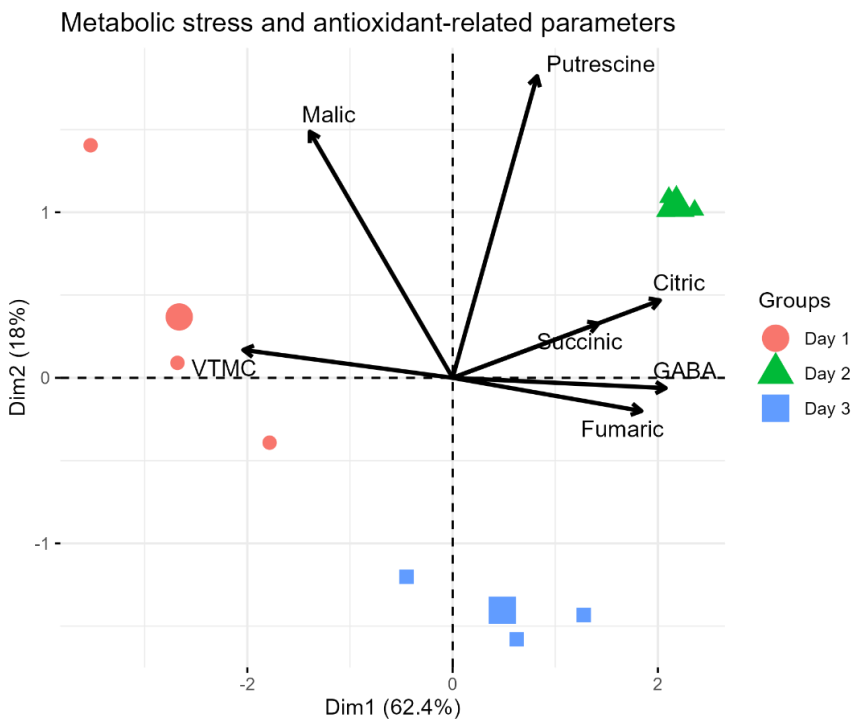

Figure 5. Principal component analysis of metabolic stress and antioxidant status during storage of iceberg lettuce (cv. Turnover)

Day 1 samples clustered on the negative side of Dim1 and were closely associated with higher vitamin C content and relatively balanced organic acid profiles. In contrast, Day 3 samples shifted toward the positive Dim1 region, characterized by lower vitamin C levels and reduced contributions from organic acids, indicating progressive metabolic decline during storage.

Day 2 samples occupied a distinct position, mainly separated along Dim2, and were associated with GABA and putrescine, suggesting activation of stress-responsive metabolic pathways. Among organic acids, citric and malic acids contributed to sample separation, whereas succinic and fumaric acids showed minimal influence, consistent with their lack of significant variation among storage days.

The spatial separation of Day 2 samples in PCA 2 reflects active metabolic reprogramming rather than metabolic deterioration. The relative stability of succinic and fumaric acids suggests maintenance of the core TCA cycle, while marked changes in malic and citric acids indicate selective regulation of carbon fluxes, particularly at branch points of the TCA cycle.

Malate and citrate serve as major carbon skeleton donors for NH_4^+ reassimilation via the GS/GOGAT pathway (Forde and Lea 2007; Masclaux-Daubresse et al. 2010). The increased demand for carbon skeletons during the NH_4^+ pulse at Day 2 likely promotes utilization of these intermediates, whereas the stable succinate–fumarate pool reflects structural integrity of the TCA cycle rather than enhanced flux. Such selective modulation of organic acid metabolism under nitrogen stress has been widely reported (Bittsánszky et al. 2015; Li et al. 2021).

The close association of Day 2 samples with GABA and putrescine further supports activation of alternative nitrogen sinks. The GABA shunt provides a rapid route for temporary nitrogen sequestration and redox stabilization, while polyamine synthesis represents a well-established protective mechanism against NH_4^+ toxicity (Forde and Lea 2007; Bittsánszky et al. 2015).

Vitamin C clustered with Day 1 samples and progressively diverged from Day 2 and Day 3, indicating antioxidant depletion during storage. This pattern is mechanistically linked to carbon metabolism, as ascorbate biosynthesis and regeneration depend on carbohydrate and organic acid availability (Smirnoff 2018). Enhanced utilization of malate and citrate for NH_4^+ detoxification, combined with increased reactive oxygen species under elevated NH_4^+, likely accelerates vitamin C consumption (Foyer and Noctor 2011; Cintya et al. 2018).

Overall, PCA 2 captures a coordinated trade-off between nitrogen detoxification and antioxidant maintenance, revealing a dynamic metabolic trajectory: Day 1 represents a homeostatic antioxidant-protected state; Day 2 reflects a transient stress-induced metabolic pulse dominated by carbon reallocation and engagement of GABA and polyamine pathways; and Day 3 marks the onset of metabolic downregulation associated with early senescence. This pattern is consistent with postharvest physiological models described by Cantwell and Suslow (2002), Chandra et al. (2008), and Amodio et al. (2018).

Based on the integrated interpretation of PCA 1 and PCA 2, a conceptual metabolic scheme is proposed to summarize the coordinated nitrogen–carbon reprogramming occurring during cold storage (Fig. 6). While PCA 1 identifies the onset of nitrogen imbalance and transient NH_4^+ accumulation as an early postharvest event, PCA 2 reveals the downstream redistribution of carbon fluxes, activation of stress-responsive pathways, and depletion of antioxidant capacity. The schematic framework links these multivariate patterns into a unified mechanistic model, illustrating how nitrogen metabolic disruption propagates into carbon metabolism, GABA and polyamine engagement, and vitamin C decline during storage.

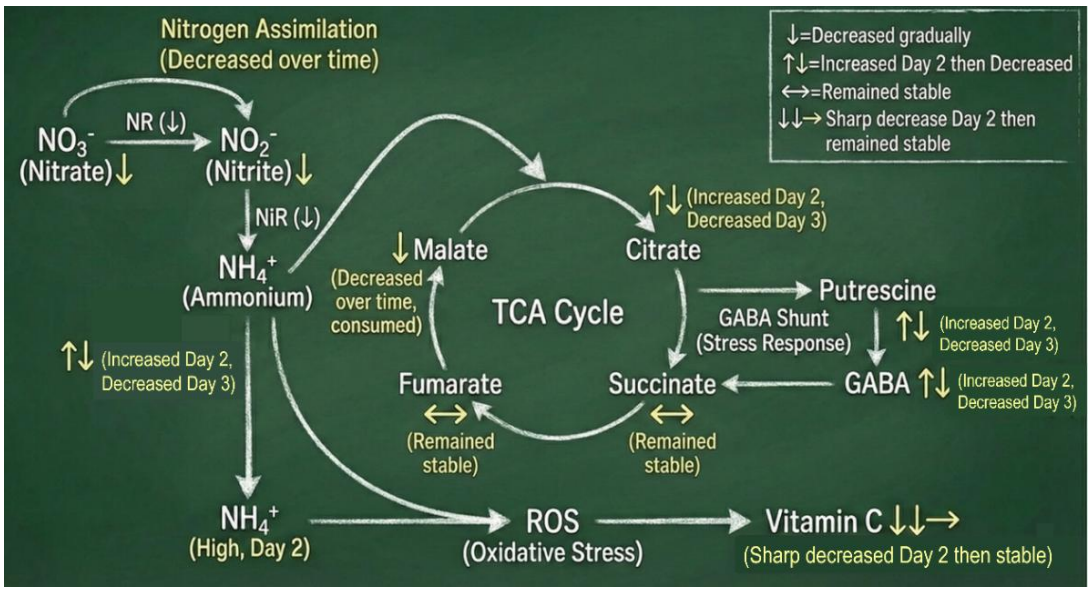

Figure 6. Metabolic responses linking nitrogen assimilation, carbon metabolism, and stress-related pathways during storage in iceberg lettuce (cv. Turnover)

The scheme integrates the multivariate patterns observed in PCA 1 and PCA 2. Early storage (Day 1) is characterized by active NR, NiR, balanced NH_4^+ assimilation, stable TCA cycle function, and high antioxidant capacity. At Day 2, partial decoupling between nitrogen reduction and NH_4^+ assimilation, combined with stress-induced protein degradation, leads to a transient NH_4^+ pulse. To mitigate NH_4^+ toxicity, carbon skeletons derived mainly from malate and citrate are redirected toward reassimilation pathways, while alternative nitrogen sinks such as the GABA shunt and polyamine (putrescine) synthesis are activated. These adjustments occur without collapse of the core TCA cycle, as indicated by stable succinate and fumarate pools. Enhanced nitrogen detoxification and redox buffering at Day 2 impose a trade-off with antioxidant maintenance, contributing to progressive vitamin C depletion. Prolonged storage (Day 3), NH_4^+ levels decline, indicating recovery of nitrogen reassimilation and metabolic stabilization.

The schematic model illustrates that short-term cold storage induces an active and transient metabolic reprogramming rather than a linear decline. Day 2 represents a critical metabolic pulse characterized by NH_4^+ accumulation, selective carbon reallocation toward citrate and malate, and activation of alternative nitrogen sinks (GABA and polyamines), at the expense of antioxidant maintenance.

CONCLUSION

This study examined whether short-term cold storage at 5 °C during the critical 72-hour logistics window can maintain metabolic stability, nutritional quality, and chemical safety in iceberg lettuce (*Lactuca sativa* L. cv. Turnover). The results demonstrate that storage under these conditions induces coordinated metabolic regulation rather than passive deterioration. Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations declined progressively, indicating sustained internal nitrogen conversion and effective detoxification through residual nitrate and nitrite reductase activity. A transient increase in ammonium (NH_4^+) on Day 2 likely reflected temporary imbalance between nitrogen release and reassimilation under cold stress, accompanied by the accumulation of stress-related metabolites (GABA and putrescine) and selective adjustments in TCA cycle–associated organic acids. By Day 3, the decline in NH_4^+ together with stabilization of vitamin C and organic acid profiles suggested recovery of metabolic balance. Importantly, NO_3^- and NO_2^- levels remained well below established safety thresholds

throughout storage. The compact head morphology of iceberg lettuce may further contribute to this stability by buffering environmental stress and preserving enzymatic functionality in inner tissues.

This study highlighted an integrated metabolic framework linking nitrogen conversion, stress-associated metabolites, carbon metabolism, and antioxidant dynamics during short-term postharvest storage. Maintaining storage at 5 °C for up to 72 h therefore represents a reliable strategy for preserving both chemical safety and metabolic integrity in iceberg lettuce. These findings provide practical insights for improving cold-chain management in Japan and regional vegetable supply systems across Southeast Asia. Integrating enzyme activity, metabolomic profiling, and storage physiology will further refine postharvest strategies aimed at optimizing both nutritional quality and food safety.

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Conceptualization: NTTN, KK; Study Design: NTTN, KK; Sample collection: NTTN, KK; Conduct of experiment: NTTN, NT; Data curation: NTTN, NT, AS, KK; Visualization: NTTN, NT, AS, KK; Formal analysis: NNTTN, NT, AS, KK; Supervision: KK; Writing – Original draft preparation: NTTN.; Writing – Review and Editing: NTTN, NT, AS, KK.