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Research Article

Ammonium Excess Promotes Proline Synthesis but Inhibits Glutathione Synthesis in Oilseed Rape (*Brassica napus* L.)

Hyunjae Lee, Seon-Hye Baek and Tae-Hwan Kim*
Department of Animal Science, College of Agriculture & Life Science, Chonnam National University, Gwangju 61186, Republic of Korea

ABSTRACT

Ammonium (NH_4^+) serves as a nitrogen source, but its elevated levels can hinder plant growth and production. Excess NH_4^+ with α-ketoglutarate is assimilated into glutamate, a precursor of proline and glutathione (GSH). This study aimed to investigate the effects of excessive NH_4^+ on the regulation of proline and GSH synthesis. Detached leaves from oilseed rape (*Brassica napus* L.) were fed with 0, 50, 100, 500, and 1000 mM NH_4Cl for 16 h. As the NH_4^+ concentrations increased, the leaves exhibited progressive wilting and yellowing. Furthermore, total carotenoid and chlorophyll concentrations declined in response to all NH_4^+ treatments, with the lowest levels observed in 1000 mM NH_4^+ treatment. Hydrogen peroxide (H_2O_2) concentration showed a minor increase at low NH_4^+ concentration (50 and 100 mM) treatments but a significant increase at high NH_4^+ (500 and 1000 mM), which was consistent with the localization of H_2O_2. Amino acid concentrations increased with increasing in NH_4^+ concentration, while the protein concentration displayed the opposite trend. Proline and cysteine concentrations exhibited a gradual increase in response to increasing NH_4^+ concentrations. However, GSH concentrations rose only in the 50 mM NH_4^+ treatment and decreased in the 500 and 1000 mM NH_4^+ treatments. These results indicate that excessive NH_4^+ is primarily assimilated into proline, while GSH synthesis is adversely affected.

(Key words: Ammonium, Glutathione, Hydrogen Peroxide, Proline)

Ⅰ. INTRODUCTION

Ammonium (NH_4^+), a major form of inorganic nitrogen (N), is taken up by plant roots and assimilated into amino acids and proteins, occurring either in roots or shoots. Initially, NH_4^+ is incorporated into glutamate through the action of glutamine synthetase, which converts it to glutamine (Castro-Rodriguez et al., 2011). Glutamine is then converted to glutamate by transferring the amide group of glutamine to α-ketoglutarate, mediated by glutamate synthase. Glutamate serves as an amino donor for the synthesis of amino acids, proteins, and nitrogen-containing metabolites (Luo et al., 2013).

When plants are exposed to environmental stress, NH_4^+ assimilation into amino acids and protein synthesis is reduced, leading to the accumulation of NH_4^+. In addition, proteins may be degraded into small peptides or amino acids (Back et al., 2022). Unused amino acids are further broken down, releasing nitrogen-containing amine groups (NH_2). These released amines can be converted back to NH_4^+, causing toxicity when NH_4^+ levels exceed a certain threshold. This toxicity can manifest as leaf chlorosis, inhibition of plant growth, and impaired root development (Shilpha et al., 2023). To mitigate NH_4^+ toxicity, NH_4^+ is assimilated into glutamate through glutamate dehydrogenase (GDH), in conjunction with α a-ketoglutarate (McAllister et al., 2012; Xian et al., 2020). It has been reported that increased GDH activity is associated with a significant accumulation of ammonia (Lutts et al., 1999). Skopelitis et al. (2006) demonstrated that glutamate is primarily produced through the GDH pathway rather than the glutamine oxoglutarate aminotransferase (GS-GOGAT) pathway under conditions where nitrogen assimilation is blocked.

Plants also promote proline synthesis to detoxify NH_4^+ under stress conditions. We have previously observed a significant positive correlation between ammonium and proline levels (Kim et al., 2004; Lee et al., 2009). Several studies have shown that proline accumulation is associated with stress tolerance. Proline maintains cell turgor, stabilizes sub-cellular

*Corresponding author: Tae-Hwan Kim, Department of Animal Science, College of Agriculture & Life Science, Chonnam National University, Gwangju 61186, Republic of Korea,
Tel: +82-62-530-2126, E-mail: grassl@chonnam.ac.kr

structures, and detoxifies reactive oxygen species (see review Hayat et al., 2012; Ghosh et al., 2021). Recently, La et al. (2020) revealed that exogenous glutamate enhances proline synthesis, resulting in improved drought stress tolerance through the activation of phytohormone salicylic acid (SA) signaling.

Glutathione (GSH) acts as an antioxidant that directly or indirectly detoxifies reactive oxygen species (ROS) and serves as a cofactor for the GSH peroxidase (Foyer and Noctor, 2011). In addition, GSH helps to protect membranes by maintaining the reduced state of compounds such as α-tocopherol and zeaxanthin (Hasanuzzaman et al., 2017). GSH, therefore, enhances plant tolerance to various environmental stresses. GSH is synthesized from three amino acids: glutamate, cysteine, and glycine. Consequently, GSH is involved in both nitrogen and sulfur assimilation and shares glutamate as a common biosynthetic precursor with proline. However, the intricate relationships between the GSH and proline synthesis under stress conditions have not been fully elucidated.

Considering that NH_4^+ is converted to glutamate under stress conditions, this study hypothesized that (1) proline and GSH biosynthesis respond differently to varying levels of exogenous NH_4^+, and (2) excess NH_4^+-induced proline accumulation may affect GSH synthesis. To test these hypotheses, we analyzed the H_2O_2 response, nitrogen assimilation, proline accumulation, and cysteine and GSH contents.

Ⅱ. MATERIALS AND METHODS

1. Plant material and NH_4Cl treatments

Oilseed rape (*Brassica napus* L. cv. Mosa) plants were grown in a greenhouse by supplying a complete nutrient solution. After eight-week, two leaves (i.e., leaf rank 5 and 6, rank one for the oldest leaf) per plant were cut at the base of the petiole and quickly rinsed with distilled water. Treatment solutions were filled into the hydroponic culture system with continuous aeration. The prepared detached leaves were immersed in water for control or different concentration of NH_4Cl solution (0, 50, 100, 500, or 1000 mM). After 16 h of treatment, leaf tissues were thoroughly rinsed with distilled water and then immediately frozen in liquid nitrogen. Afterward, samples were stored in a deep freezer for further analysis.

2. Measurement of total carotenoid and chlorophyll concentrations

Fresh leaves (100 mg) were immersed in 10 mL of dimethyl sulfoxide at 65℃ for 1 h and then read the absorbance at 480 and 510 nm for carotenoid, and 645 and 663 nm for total chlorophyll concentrations, respectively. The concentrations were calculated with a formula described by Kim et al. (2022).

3. Determination of H_2O_2 localization and concentration

H_2O_2 histochemical localization was detected after immersing leaf discs in sodium phosphate buffer (pH 7.0) containing 3,3-diaminobenzidine tetrahydrochloride (1 mg per mL). The reaction was terminated when dark spots appeared. The stained leaves were photographed after bleaching in boiling ethanol (Lee et al., 2013). H_2O_2 concentrations were quantified according to the method of Junglee et al. (2014). Fresh tissues were extracted with 0.1% trichloroacetic acid and centrifuged at 12,000 × g for 10 min at 4℃. The 50 μL of supernatant reacted with 100 μL of 1M potassium iodide and 50 μL of 10 mM potassium phosphate buffer (pH 5.8), incubated for 30 min, and then the absorbance was measured at 390 nm.

4. Chemical analysis

Amino acids and protein concentrations were measured according to the method of Back et al. (2022). For proline concentration, fresh leaves (200 mg) were extracted with 3% sulfosalicylic acid. After centrifugation, the supernatants were mixed with ninhydrin solution, boiled for 60 min at 70℃, and then added to toluene. The absorbance was determined at 520 nm. Cysteine concentration was determined using the method of Lee et al. (2022). Fresh leaves (200 mg) were extracted with sodium phosphate buffer (pH 7.5) and centrifuged at 12,000 × g for 10 min at 4℃. The supernatant was mixed with 2.5% ninhydrin solution in acetic acid and HCl (6:4, v/v) and boiled for 10 min. The absorbance was read at 560 nm. Glutathione (GSH) concentration was measured using GSH/GSSG Kit GT40 (Oxford Biomedical Research Inc.).

5. Statistical analysis

Duncan's multiple range test was employed to compare the means of separate replicates. All statistical tests were performed using ASA 9.4 (SAS Institute Inc., Cary, NC, USA).

Ⅲ. RESULTS

1. Phenotypes and pigment concentrations in detached leaves

As NH_4^+ concentrations increased, observable effects such as leaf wilting and yellowing became more prominent (Fig. 1A). Total carotenoid concentration showed a consistent decrease regardless of NH_4^+ concentrations (Fig. 1B). Furthermore, total chlorophyll concentrations gradually declined with higher levels of exogenous NH_4^+ (Fig. 1C).

2. H_2O_2 localization and concentration in detached leaves

H_2O_2 has slightly detected up to 100 mM NH_4^+ treatment, but became more pronounced in the 500 and 1000 mM NH_4^+ treatments, leading to brown spots on the leaves (Fig. 2A). The concentrations of H_2O_2 were continuously increased up to the 500 mM NH_4^+ treatment (+33.4% compared to the 0 mM NH_4^+ treatment) and sustained at the 1000 mM NH_4^+ treatment (Fig. 2B).

3. Amino acid and protein concentrations in detached leaves

A significant increase in amino acid concentration was observed from 50 mM NH_4^+ treatment, reaching a maximum level at the 1000 mM NH_4Cl treatment (+6.5-fold increase compared to the 0 mM NH_4^+ treatment) (Fig. 3A). In contrast, protein concentration gradually decreased up to the 500 mM NH_4^+ treatment (-57.3% compared to the 0 mM NH_4^+ treatment) and remained constant in the 1000 mM NH_4^+ treatment (Fig. 3B).

4. Proline, cysteine, and glutathione concentration in detached leaves

Proline concentrations showed no significant change in the 50 mM NH_4^+ treatment but exhibited substantial increases of 3.4- and 3.7-fold in the 500 and 1000 mM NH_4^+ treatments,

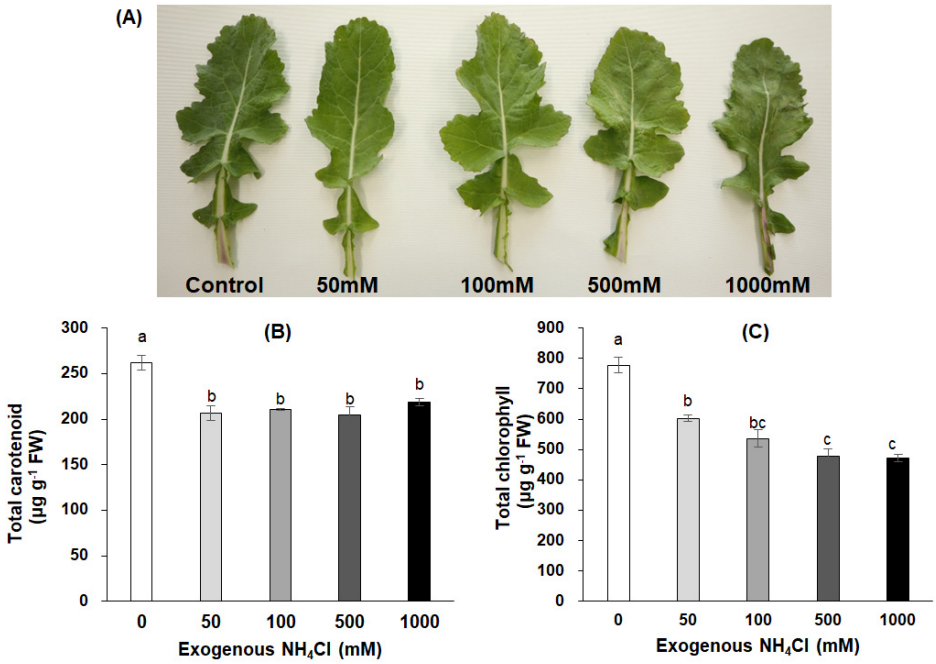

Fig. 1. Effect of exogenous NH_4Cl on phenotypes (A) and total carotenoid (B) and chlorophyll (C) concentrations in detached leaves of *Brassica napus*. Data are represented as mean ± s.e. for n=3. According to Duncan's multiple range test, bars labeled with different letters are significantly different (p<0.05).

respectively (Fig. 4A). Similarly, cysteine concentration gradually increased up to the 1000 mM NH_4^+ treatment (Fig. 4B). In contrast to cysteine, GSH concentration decreased significantly with increasing NH_4^+ concentration, except in the 50 mM NH_4^+ treatment (Fig. 4C).

Ⅳ. DISCUSSION

In this study, we found that excess of NH_4^+ led to NH_4^+ toxicity, such as leaf wilting and yellowing, accompanied by a decrease in total chlorophyll and carotenoid concentrations (Fig. 1). These findings align with previous studies conducted on tobacco (Skopelitis et al., 2006) and barley (Coskun et al., 2013). The reduction of chlorophyll levels can be attributed to decreased nitrogen accumulation in shoots, which is essential for chlorophyll formation (Wang et al., 2014). Furthermore, high NH_4^+ levels were found to suppress photosynthesis by downregulating genes associated with light-harvesting chlorophyll a/b binding, disrupting the photochemistry of chloroplast and leading to increased generation of ROS such as H_2O_2 and O_2^- (Yang et al., 2020).

The results regarding H_2O_2 accumulation were consistent with the histochemical observations of H_2O_2 distribution (Fig.

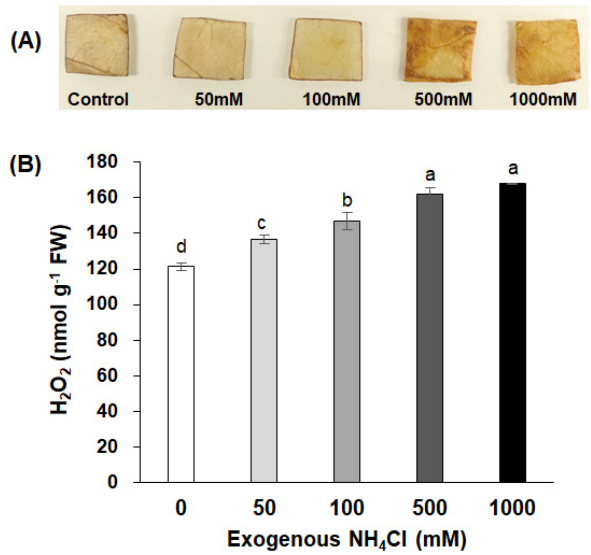

Fig. 2. Effect of exogenous NH_4Cl on histochemical localization of H_2O_2 through DAB staining (A) and H_2O_2 concentrations (B) in detached leaves of *Brassica napus*. Data are represented as mean ± s.e. for n=3. According to Duncan's multiple range test, bars labeled with different letters are significantly different (p<0.05).

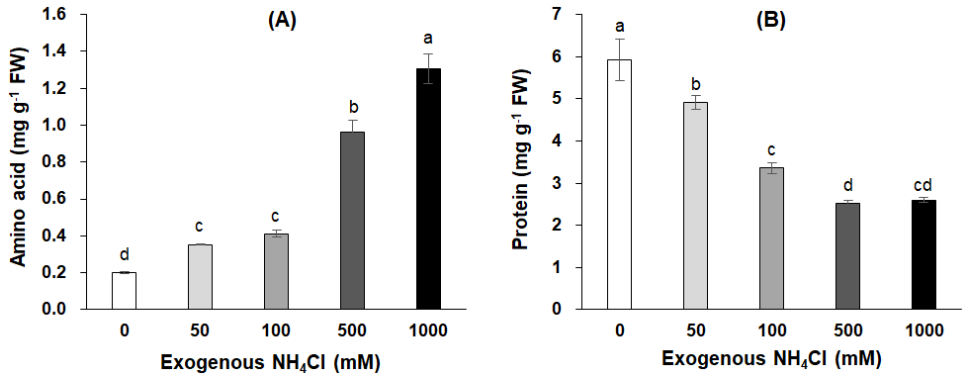

Fig. 3. Effect of exogenous NH_4Cl on amino acid (A) and protein (B) concentrations in detached leaves of *Brassica napus*. Data are represented as mean ± s.e. for n=3. According to Duncan's multiple range test, bars labeled with different letters are significantly different (p<0.05).

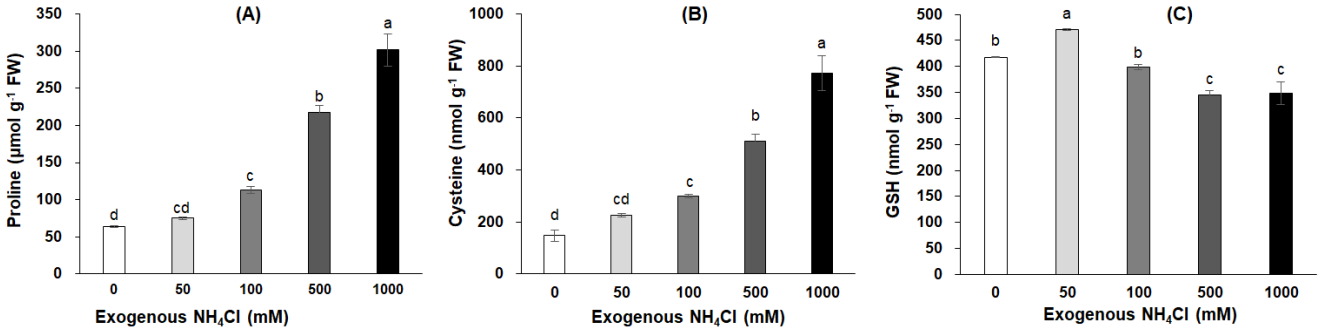

Fig. 4. Effect of exogenous NH_4Cl on proline (A), cysteine (B), and glutathione (GSH, C) concentrations in detached leaves of *Brassica napus*. Data are represented as mean ± s.e. for n=3. According to Duncan's multiple range test, bars labeled with different letters are significantly different (p<0.05).

2). Similar studies have reported an increase in lipid peroxidation and activity of enzymes involved in the ascorbate-glutathione (AsA-GSH) cycle under NH_4^+ treatment (Lee et al., 2013). In rice, an elevation in GSH production has been associated with the activation of NH_4^+-induced ROS scavenging, accompanied by upregulation of glutathione reductase gene expression (Yang et al., 2020). In contrast to these results, our study revealed that high NH_4^+ treatment (500 and 1000 mM) decreased GSH concentration, while low NH_4^+ treatment (50 mM) resulted in an increase (Fig. 4C). A reduction in GSH concentration has also been observed in *Brassica napus* under drought stress (La et al., 2019), suggesting that GSH levels are influenced by stress intensity and species.

Our previous research found that exogenous NH_4^+ led to an accumulation of ammonia and increased proline content in detached white clover leaves (Lee et al., 2013). In addition, we demonstrated that drought stress-induced reduction of nitrogen assimilation caused an increase of NH_4^+, leading to the accumulation of proline, as indicated by ^{15}N-isotope analysis (Kim et al., 2004; Lee et al., 2009). These findings have also been confirmed in rice under conditions of NH_4^+ accumulation caused by methionine sulfoximine [glutamine synthetase (GS) inhibitor]-induced inhibition of nitrogen assimilation (Yang and Kao, 2000; Barth et al., 2010). In our current study, high NH_4^+ treatment also resulted in a reduction of protein concentration, which in turn led to increased amino acid concentrations (Fig. 3), a common response for protecting nitrogen assimilation under various stresses. Considering the reduction in GS and GOGAT activities under the NH_4^+ excess condition (Yang et al., 2020), the increased amino acids may be attributed to protein degradation by protease. Carr et al. (2020) also reported that higher amounts of lysine, methionine, cysteine, and proline were presented in higher NH_4^+-treated plants. Our data also showed a gradual increase in cysteine and proline concentrations according to the NH_4^+ level (Fig. 4A and B).

Proline is synthesized from glutamate via the intermediated pyrroline-5-carboxylate (P5C) which is catalyzed by pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). Therefore, the proline accumulation under NH_4^+ excess conditions may result from the accumulation of glutamate under higher NH_4^+ conditions. We previously found that exogenous glutamate promotes proline synthesis by activating the expression of the *P5CR* gene in *Brassica napus* (La et al., 2020). Moreover, a positive correlation between proline and NH_4^+ has been observed in white clover (Kim et al., 2004; Lee et al., 2013). On the other hand, GSH synthesis from glutamate increased under low NH_4^+ conditions but decreased under NH_4^+ excess (Fig. 4C). During the initial stages of stress, plants promote activities of ascorbate peroxidase and glutathione reductase enzyme to scavenge ROS, especially H_2O_2, resulting activation of AsA-GSH cycle (Foyer and Noctor, 2011). Therefore, an increase in GSH at low NH_4^+ may be closely related to the detoxification of ROS. These findings suggest that proline and GSH concentrations are synergistically increased to scavenge H_2O_2 at low NH_4^+ conditions, whereas proline is predominantly accumulated under NH_4^+ excess.

Ⅴ. ACKNOWLEDGEMENTS

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